

WORLD HEALTH ORGANIZATION



INTERNATIONAL AGENCY FOR RESEARCH ON CANCER

**RELEVANCE OF N-NITROSO  
COMPOUNDS TO HUMAN CANCER:  
EXPOSURES AND MECHANISMS**

*Proceedings of the IXth International Symposium  
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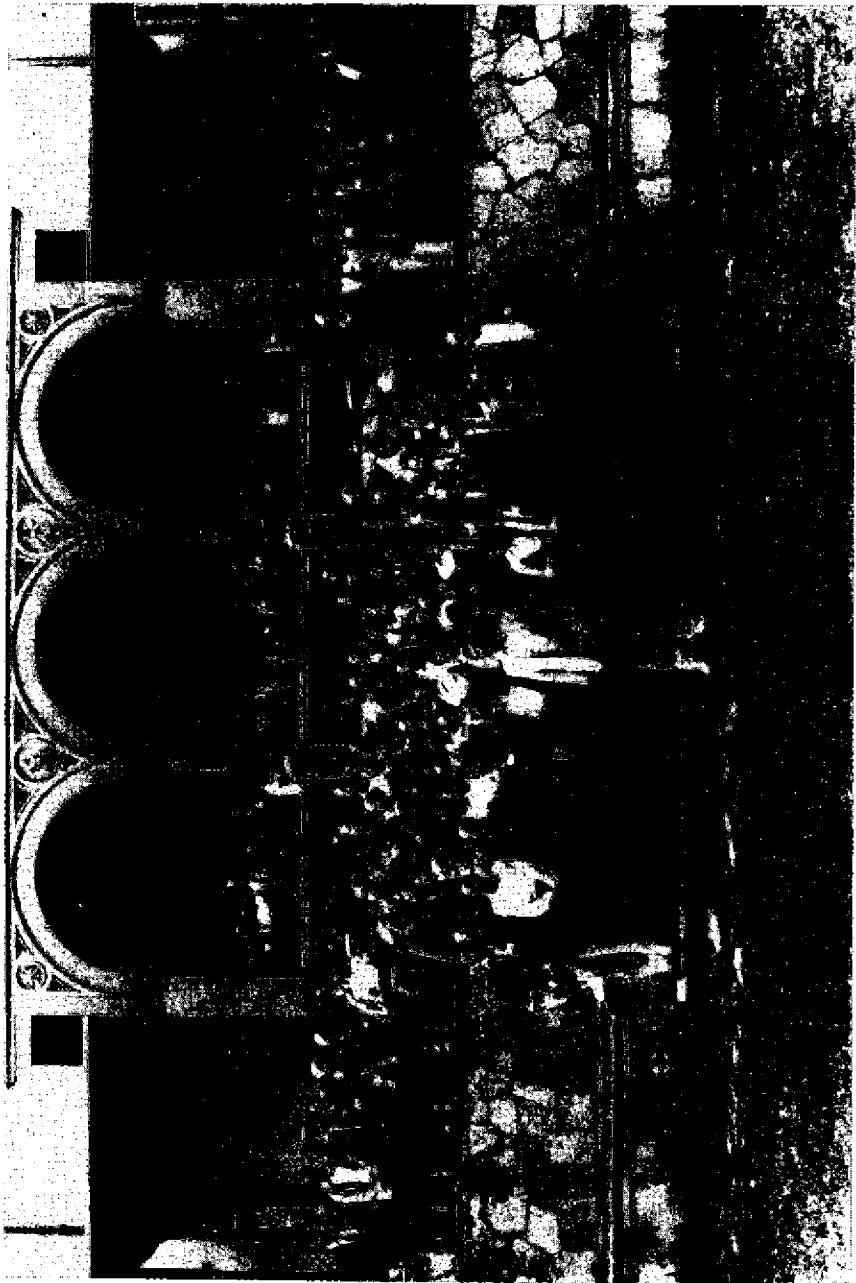
## FOREWORD

This volume is the proceedings of the Ninth International Meeting on *N*-Nitroso Compounds, held in Baden, Austria. Many of the contributions reflect recent interest in endogenous formation of these compounds in humans and the need both to elucidate the microbiological and dietary conditions that affect this source of exposure and to establish a link with DNA damage and increased cancer incidence. In several studies, the potential connection between oral cancer and *N*-nitroso compounds in chewing tobacco was investigated further. The carcinogenicity of *N*-nitroso compounds in all of the 40 animal species so far tested and their ubiquitous occurrence support the hypothesis, which must now be tested, that they may have an etiological role in several other human cancers, such as those of the oesophagus, stomach, urinary bladder, pancreas and brain.

*N*-Nitroso compounds continue to serve as valuable tools in cancer research, not least in reflecting a complex system of formation and metabolism that is consistent with the multifactorial causes of non-occupational, as well as occupational, cancers.

I should like to thank the Programme Committee for their work and the Government of Austria, the US National Cancer Institute and Dr M. Okada (Tokyo Biochemical Institute) for sponsoring and supporting this meeting.

Lorenzo Tomatis, MD  
Director  
International Agency for Research on Cancer



## INTRODUCTION

The present volume, the proceedings of the Ninth International Meeting on *N*-Nitroso Compounds<sup>1</sup>, reflects, in its title and in the large number of participants present, interest in assessing the relevance of *N*-nitroso compounds to human cancer. IARC celebrated its 20th anniversary in 1986, and this series of meetings was initiated by Dr P. Bogovski, soon after the foundation of the Agency. These meetings are an example of successful interaction between experimentalists of different backgrounds and scientific specialities and epidemiologists — the type of collaboration the Agency has fostered since its inception. Indeed, as reported at this ninth conference, an increasing number of biochemical and epidemiological surveys tried to identify carcinogenic hazards and to indicate variables that may entail a reduction in exposure/risk (see Overview, p. 5).

With respect to our host country and host institution, an eminent scientist from the University of Vienna, Erwin Chargaff, should be remembered. He graduated from the University of Vienna in 1927 and later became a Professor at Columbia University in New York. He made outstanding contributions to DNA chemistry as early as 1949; many articles in this volume deal with the interaction of *N*-nitroso carcinogens with DNA as a key to understanding mechanisms of action and for assessing individual exposure.

The fact that the ninth nitrosamine conference was a successful one was due largely to the highly qualified audience, chairpersons and programme committee. The Editors wish to thank all for their contributions, guidance and assistance.

The Editors

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<sup>1</sup>The tenth meeting is planned to be held in September 1989 in Beijing, China.

## **N-NITROSO COMPOUNDS: EXPOSURES, MECHANISMS AND RELEVANCE TO HUMAN CANCER — AN OVERVIEW<sup>1</sup>**

The ninth meeting of this series was attended by 200 participants from 24 countries, who presented papers and review lectures focusing exclusively on NOC<sup>2</sup> and their precursors. The keynote address (p. 11) outlined the usefulness of NOC for studying basic mechanisms of carcinogenesis; it was stressed that available biochemical and histopathological data give little reason to believe that humans are resistant to the carcinogenic action of NOC. Sequential steps were reviewed in hepatocarcinogenesis, which is frequently induced by NOC, and two concepts related to the mechanism of tumour promotion were described (p. 17): the 'resistant cell' hypothesis and that of 'overexpression' of adaptive growth.

### **1. Macromolecular adducts, DNA repair and biological consequences**

Models for studying *in vitro* the effects of NOC in human tissues and cells were reviewed (p. 20). *N*-Nitrosamines are metabolically activated by cultured human cells to yield DNA-damaging metabolites including alkyldiazonium ions and aldehydes. These metabolites can also inactivate DNA repair processes and are mutagenic in human cells. Both human epithelial and mesenchymal cells have been transformed *in vitro* by NOC. This process may involve mutations of oncogenes. The human *c-Ha-ras-1* proto-oncogene is shown to be converted into a transforming oncogene after reaction *in vitro* with *N*-nitrosomethylacetoxymethylamine as revealed by transfection into NIH 3T3 cells (p. 26). *O*<sup>6</sup>-Alkylguanine residues in DNA are repaired by AAT, for which the preferred substrate is *O*<sup>6</sup>-meGua, higher alkylhomologues being repaired at a progressively slower rate (p. 30). <sup>32</sup>P-Labelled synthetic oligodeoxynucleotides containing *O*<sup>6</sup>-meGua provide ultra-sensitive assays for AAT, the levels of which are found to vary greatly between different tissues and species (p. 30; p. 41). Animals fed diets restricted in the essential amino acid cysteine have delayed repair of *O*<sup>6</sup>-meGua, ascribed to a diminished level of *O*<sup>6</sup>-AT in the liver (p. 35). Using immunocytochemical visualization of *O*<sup>6</sup>-alkylguanine in liver DNA of rats treated with *N*-ethyl-*N*-nitrosourea, NDEA or NDMA, large differences in the level of formation repair of *O*<sup>6</sup>-alkylguanine are found between different cell types, between hepatocytes in different locations, and also between normal and precancerous hepatocytes (p. 55). The relationships are examined between *O*<sup>6</sup>-ethyldeoxy-

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<sup>1</sup> This overview is partially based on summaries prepared by the session chairmen of the meeting: P. Bogovski (Institute of Experimental & Clinical Medicine, Tallinn, USSR), M. Börzsönyi (National Institute of Hygiene, Budapest, Hungary), B.C. Challis (Imperial College, London, UK), G. Eisenbrand (University of Kaiserslautern, FRG), D. Hoffmann (Naylor Dana Institute for Disease Prevention, Valhalla, NY, USA), P. Magee (Fels Research Institute, Philadelphia, PA, USA), M. Okada (Tokyo Biochemical Research Institute, Tokyo, Japan), R. Preussmann (German Cancer Research Center, Heidelberg, FRG), S.R. Tannenbaum (Massachusetts Institute of Technology, Cambridge, MA, USA).

<sup>2</sup> The abbreviations used are: NOC, *N*-nitroso compound; NDMA, *N*-nitrosodimethylamine; NPRO, *N*-nitrosoproline; NDEA, *N*-nitrosodiethylamine; NDELA, *N*-nitrosodiethanolamine; NNK, 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*'-nitrososornicotine; *O*<sup>6</sup>-meGua, *O*<sup>6</sup>-methylguanine; AAT, *O*<sup>6</sup>-alkylguanine transferase

thymidine levels, tumour occurrence and the number of  $\gamma$ -glutamyl transferase positive foci in the livers in rats treated with NDEA (p. 59). *O*<sup>6</sup>-Ethyldeoxythymidine accumulates in DNA of hepatocytes, and lobes respond heterogeneously with regard to cell proliferation and hepatocarcinogenesis. Further proof of the mutagenicity of *O*<sup>6</sup>-methyl- and *O*<sup>6</sup>-ethyldeoxythymidine comes from site-directed mutagenesis experiments in which deoxythymidine is replaced by *O*<sup>6</sup>-alkyldeoxythymidine. All sequenced revertants have adenine  $\rightarrow$  guanine transitions (p. 37). Some insights into the biological basis of organ-specific carcinogenesis by asymmetrical *N*-nitrosomethylalkylamines are presented (p. 49): after chronic exposure, DNA was determined in target and nontarget tissues, and the levels were correlated with tumour induction in the oesophagus.

## 2. Metabolism and modifying factors

$\beta$ -Hydroxynitrosamines can be activated metabolically in at least three different ways:

(a) by  $\alpha$ -hydroxylation. Rat liver DNA is methylated and 2-hydroxylated *in vivo* by *N*-nitrosomethyl(2-hydroxymethyl)amine, methylation being clearly favoured over 2-hydroxylation (p. 77). Interaction of the metabolites of NDELA with rat liver DNA results in the formation of *N*7-(2-hydroxyethyl)guanine and *O*<sup>6</sup>-(2-hydroxyethyl)guanine in nucleic acid hydrolysates (p. 87). *N*-Nitrosamino- $\alpha$ -phosphates have been synthesized and shown to be mutagenic; they could act as transport forms of activated nitrosamines *in vivo* (p. 162; p. 165).

(b) by sulfate conjugation (p. 77; p. 83). Strong evidence that metabolic activation of  $\beta$ -hydroxynitrosamines to genotoxic metabolites involves conjugation with sulfate has been provided. Thus, inhibition of sulfotransferases by 2,6-dichloro-4-nitrophenol *in vivo* prevents DNA single-strand breaks induced by NDELA and its metabolite *N*-nitroso-2-hydroxymorpholine.

(c) by  $\alpha$ -nitrosamino aldehyde formation (p. 94).  $\beta$ -Hydroxynitrosamines undergo lactate dehydrogenase-catalysed oxidation to the corresponding aldehydes, which are highly reactive compounds and directly-acting mutagens, and which are capable of transnitrosation to secondary and primary amines. *N*-Nitroso-*N*-butyl-2-ethanolamine undergoes spontaneous decomposition to yield butyl diazonium ion, or it can react with guanosine to produce xanthosine (by deamination), 7-butylguanosine and the 1,*N*<sup>2</sup>-glyoxal adduct.

Metabolic denitrosation of nitrosamines has now been demonstrated in several studies (p. 113; p. 117). In rats, NDMA undergoes enzymatic cleavage to nitrite and dimethylamine, which are excreted in the urine at roughly equimolar concentrations. Denitrosation was also demonstrated *in vivo* using <sup>15</sup>*N*-nitroso-*N*-methylaniline and *N*-nitrosodiphenylamine. Both nitrate and nitrite are found as metabolic products when *N*-nitrosamines are incubated with a rat-liver microsomal system. The NADPH-dependent microsomal denitrosation of NDMA has been investigated with a new procedure in which nitric oxide is determined under aerobic conditions (p. 109); nitric oxide is formed as a precursor of nitrite by a superoxide dismutase-insensitive reaction. The enzyme involved is the cytochrome P450 isozyme responsible for the dealkylation of NDMA. The metabolism of several *N*-nitrosoalkylamines has been studied (p. 104) using purified P450 isozymes in a reconstituted monooxygenase system. P450 (ethanol-inducible) showed high NDMA demethylase activity, and a close relationship between the demethylation and denitrosation of the substrate was observed.

A number of studies are presented on cell-type- or organ-specific differences in the metabolic activation of nitrosamines that add new information about the underlying mechanisms by which nitrosamines produce tumours in different organs and species. The pig and the ferret can be used as models for simulating the endogenous synthesis and metabolism of nitrosamines in humans (p. 132; p. 135). *N*-Nitrosodibutylamine forms alkylating electrophiles *in vivo* that can be detected as their *N*-acetyl-*S*-butyl-L-cysteine derivatives in the urine of rats (p. 153).

Several studies examine the effect of modifiers on the metabolism, the mutagenicity and the DNA binding of NOC. For example, ellagic acid inhibits the mutagenic activity of *N*-methyl-*N*-nitrosoarene and selectively inhibits *O*<sup>6</sup>-methylation of guanine in double-stranded DNA (p. 197). Metabolism of the oesophageal carcinogen *N*-nitrosopentylmethylamine by excised oesophagus from rats and hamsters is shown to be dependent on the age of the animals: it was fastest during the first ten days of life and declined with age (p. 144).

Ethanol has been found to increase the carcinogenicity of several nitrosamines in the mouse forestomach (p. 264). Zinc-deficient rats given a single intragastric dose of *N*-methyl-*N*-nitrosoarene developed malignant lymphomas (p. 261).

Experiments to investigate syncarcinogenesis using combinations of different chemical carcinogens and modifiers in rats and mice are reviewed (p. 246). In a recent study in which rats received a combination of NDEA, *N*-nitrosopyrrolidine and NDELA at low doses, the incidence of liver tumours increased in an additive fashion.

### 3. Methods for detection and measurement of exposure

A new technique, using laser photofragment spectroscopy, has been developed for the detection and discrimination of *N*-nitrosamines, alkylnitrites and *C*-nitroso compounds. This technique is more sensitive than those using the Thermal Energy Analyzer and shows promise for the characterization of volatile and nonvolatile NOC (p. 228). An *in-vitro* assay is described (p. 232) for detecting alkylating and mutagenic activities of dietary components nitrosated *in situ*; it combines the 4-(*para*-nitrobenzyl)pyridine colorimetric test with the Ames assay.

Magnetic semipermeable polyethyleneimine microcapsules have been devised for trapping and analysing nitrosating and alkylating species formed in the gastrointestinal tract (p. 222).

Determination of alkylated nucleic acid bases in urine may be useful for estimating the burden of DNA alkylation *in vivo* arising from exposure to NOC. Excretion of 3-methyl adenine was found to be a good indicator of endogenous nitrosation of drugs that yield alkylating agents (p. 407). 7-Carboxymethylated guanine has been detected in the urine of animals fed *N*-nitrosoglycocholic acid (p. 187).

### 4. Experimental studies on formation

Endogenous formation of NOC, particularly by intragastric nitrosation, is suspected to play a role in the etiology of human cancers. Compounds present in normal gastric juice of fasting humans can apparently be nitrosated to produce alkylating agents that are detectable as adducts after incubation with substrate DNA (p. 507). Other physiological constituents, such as peptides, can be converted *in vitro* into mutagenic diazo- or *N*-nitrosopeptides (p. 308), although there is no direct evidence for their formation *in vivo*. Other recently identified nitrite-reactive compounds consumed by humans, and the biological properties of their nitrosated products, are summarized (p. 287). Tyramine and  $\beta$ -carboline derivatives isolated from soya sauce and some substituted indole derivatives isolated from Chinese cabbage have been found to be mutagen precursors. Following reaction with nitrite, these compounds produce directly-acting mutagens, among which 3-diazotyramine was also shown to be carcinogenic to rats.

Glycosylamines and Amadori compounds present in food or formed during early stages of nonenzymic browning (Maillard) reactions were nitrosated and the properties of the resulting NOC investigated. Several members of this class show high mutagenicity and induce DNA damage in mammalian cells; a mechanism for the formation of alkylating agents has been elucidated and structure-activity relations established (p. 274; p. 277).

Bacterially-mediated nitrosation can take place at neutral pH. Several strains, including *Neisseria mucosa*, *Pseudomonas aeruginosa* and *Escherichia coli*, isolated from human gastric microflora and infected human organs, exhibit high nitrosating activity when incubated with nitrite and a secondary amine (p. 391; p. 396; p. 400). Infection of the urinary bladder with *Escherichia coli* gives rise to tumours in rats when secondary amines and nitrates are administered (p. 380) and also augments tumour production by the bladder carcinogen *N*-nitroso-*N*-butyl-*N*-(4-hydroxybutyl)amine. Strains of bacteria isolated from human subjects with urinary-tract infections catalyse nitrosamine formation, and urine from such patients contains elevated levels of volatile and nonvolatile NOC (p. 384).

A new pathway of potentially great importance for the formation of NOC in humans is that mediated by macrophages, which proliferate in response to stimulation by *E. coli* lipopolysaccharide and interferon and produce nitrite and nitrate (p. 335). Moreover, nitrosating intermediates are produced that are capable of forming NOC (p. 340). Thus, nitrosamine formation can occur at physiological pH at sites remote from the stomach, and such macrophage-mediated nitrosation reactions could be of importance in infection and inflammatory diseases.

### 5. Endogenous formation in humans

Human exposure to nitrosating agents and precursors can lead to endogenous synthesis of nitrosamines by multiple pathways (p. 292). Reaction of  $\text{NO}_x$  in the lung, acid-catalysed nitrosation in the stomach, and nitrosation at neutral pH mediated by bacteria and macrophages at other sites of the body, appear to be the nitrosation reactions most relevant to humans. Inhibition of the endogenous nitrosation of proline in human volunteers requires at least a 2:1 molar ratio of ascorbic acid to nitrite (p. 299).

Many investigators have examined the model of human gastric cancer that is based on in-vivo nitrosation. There is now good agreement on several steps in the model, elucidated by studies on patients with chronic atrophic gastritis or pernicious anaemia or who have undergone gastrectomy, all of whom are at higher risk of developing stomach cancer. These subjects tend to have a higher than normal gastric pH, which leads to bacterial overgrowth and nitrite production in the stomach (p. 511; p. 527; p. 531). There is still controversy, however, about whether subsequent *N*-nitrosation takes place and how it is related to pH or to bacterial catalysis. Two studies clearly show that urinary NPRO is negatively correlated with gastric pH, nitrite concentration and number of nitrate-reducing bacteria in fasting gastric juice (p. 511; p. 527). Most of the controversy arises, however, from differences in the methods used for the analysis of total NOC in gastric juice. An improved procedure has been validated (p. 209) which appears to discriminate between NOC and other compounds in gastric juice that respond in the Thermal Energy Analyzer.

### 6. Tobacco and betel-quid carcinogenesis

Evidence for the involvement of NOC in tobacco-related tumours is becoming stronger. Several tobacco-specific nitrosamines, such as NNK and NNN, give rise to characteristic oral, nasal and respiratory-tract tumours in animals. Moreover, NNK and NNN form haemoglobin adducts in rats ( $t^{1/2}$ , one to two weeks); hydrolysis of these adducts releases several compounds, including 4-hydroxy-1-(3-pyridyl)-1-butanol, which has been detected in some tobacco smokers. Methods for the analysis of other markers of exposure to tobacco-specific carcinogens, including DNA adducts derived from NNK and NNN, are being developed using a  $^{32}\text{P}$ -postlabelling assay and immunoassays for *O*<sup>6</sup>-meGua; although the latter adduct was expected to be formed, it was not detected in exfoliated oral cells from snuff dippers (p. 423).

Molecular dosimetry has been carried out (p. 430) on *O*<sup>6</sup>-meGua in DNA from lung and nasal mucosa of rats treated with NNK. The dose-response curve for NNK is nonlinear in the respiratory mucosa and linear in olfactory mucosa. *O*<sup>6</sup>-meGua levels are much higher in respiratory epithelium, where Clara cells contain the highest amount. The majority of nasal tumours induced by NNK appear to originate in the olfactory region, suggesting that both formation of premutagenic adducts and cell proliferation are required for the initiation of neoplasia within the nose. This hypothesis was confirmed (p. 434) using high-resolution microautoradiography for detection of bound radioactivity in rats given NNN, which shows labelling in the olfactory region of the nose (secretory cells of Bowman's glands) and in the oesophagus (squamous epithelium). Both sites are targets for the carcinogenicity by NNN; binding of metabolites in other tissues (trachea and lung), however, does not correlate with carcinogenic responses.

Preneoplastic cellular changes induced by NNK in the respiratory tract of Syrian golden hamsters are reported to be similar to those observed in the tissues of smokers (p. 438). Exposure to NNK may also be related to the occurrence of chronic bronchitis and interstitial pneumonitis, which are often observed in smokers.

Studies aimed at showing that saliva of betel quid chewers contains nitrosamines derived from areca nut alkaloids are reviewed; the saliva of chewers of betel quid containing tobacco also contains tobacco-specific nitrosamines (p. 465). By adding proline to the betel-quid mixture and then analysing the saliva of chewers for NPRO, it has been demonstrated that some of these nitrosamines are formed during chewing.

Traces of 3-(nitrosomethylamino)propionitrile, a new arecoline-derived nitrosamine, have been reported in the saliva of betel-quid chewers (p. 456). This compound is a powerful carcinogen in rats, inducing cancer of the nasal cavity and, at high doses, benign tumours of the oesophagus and tongue. DNA from the nasal cavity of these rats contains *N*7- and *O*<sup>6</sup>-meGua, as well as an unknown adduct. In addition to the four tobacco-specific nitrosamines identified earlier in snuff, two new ones have been found, at ppm levels. After oral administration of mixtures of NNN and NNK, rats develop tumours of the oral cavity and lung, showing that these tobacco-specific nitrosamines can also induce local tumours. Tobacco-related aldehydes (formaldehyde, acetaldehyde, acrolein) produce DNA-protein cross-links and decrease the thiol content in cultured human fibroblasts and xeroderma pigmentosum cells. These aldehydes also inhibit AAT activity; and formaldehyde potentiates the mutagenicity of *N*-methyl-*N*-nitrosourea (p. 443).

The effects have been examined of cigarette smoking and dietary factors on urinary excretion of NPRO and other nitrosamino acids (p. 446). The levels excreted by cigarette-smoking volunteers on a fixed diet are about double those of nonsmokers, and major increases in the urinary excretion of these nitrosamino acids are found in subjects who have eaten certain Japanese foods (*tara chiru*), probably due to their high content of nitrosamine precursors.

## 7. Clinical and epidemiological studies

In order to assess the role of NOC in human disease, a large epidemiological study is being conducted to establish the etiology of childhood brain tumours (p. 477). Maternal and paternal exposure to NOC is being assessed retrospectively using a comprehensive questionnaire.

In a case-control study, the daily consumption in food of precursors of nitroso compounds and of protective agents was compared with the incidence of gastric cancer (p. 492). Average daily consumption of nitrite and carbohydrate is associated with an increasing trend in risk, while citrus fruit intake appeared to be somewhat protective. Increased consumption of dietary fibre was found to reduce gastric cancer risk. Studies of precursor lesions for gastric cancer in



populations in Colombia and Louisiana (USA) show that gastric cancer has a complex etiology involving genetic components (p. 485). Several steps of the model for gastric carcinogenesis that is based on in-vivo nitrosation have been explained, but controversy remains with regard to which factors (nitrate, intragastrically formed NOC, infection by *Campylobacter pyloridis*) are involved in the causation of superficial or atrophic gastritis, leading to achlorhydria.

Inhabitants of high- and low-risk areas for stomach cancer in Japan were examined after intake of proline (p. 497). Urinary NPRO increases significantly only in subjects from the high-risk area, and vitamin C inhibited this increase. Urinary levels of nitrate are higher in subjects from the low-risk area, and the levels correlate with the amount of vegetables consumed. The fact that the potential for intragastric nitrosation of high risk subjects is elevated suggests that intake of inhibitory components in the diet is an important factor in the low-risk area. These data also indicate that nitrate/nitrite measurements in saliva, urine, gastric juice and blood are not sufficient to predict the outcome of the complex nitrosation process in humans. Studies have also been conducted on the association between risk for cancer of the oesophagus and levels of nitrosamino acids in the urine including a correlation study of urinary excretion of nitroso compounds and cancer mortality in 26 counties in China (p. 503). Oesophageal cancer mortality rates appear to be associated positively with endogenous formation of NPRO and negatively with plasma ascorbic acid levels. In a second study, inhabitants of high-risk areas for oesophageal cancer in China were shown to have elevated urinary excretion of nitrosamino acids (p. 538). In addition, higher levels of *N*-nitroso-methylbenzylamine, a known oesophageal carcinogen, were detected in the gastric juice of inhabitants of high-risk areas than in those of people from low-risk areas. Also, in cancer patients from a high-risk population (Linxian county, China), elevated levels of the promutagenic lesion *O*<sup>6</sup>-meG were detected in DNA of oesophageal epithelial cells, while the repair capacity for this lesion in oesophageal tissue appears to be normal (p. 534).

In Thailand, levels of excretion of total nitrate and NPRO were higher in subjects at high risk for cholangiocarcinoma, who are infected with the liver fluke (*Opisthorchis viverrini*) (p. 544). Convincing evidence is presented of a causal association between intake of Cantonese-style salted fish during childhood and nasopharyngeal carcinoma in young Chinese (p. 547). In experiments in progress in which rats are fed the same type of salted fish, some animals developed nasal cavity carcinomas after 98 weeks. Whether preformed volatile nitrosamines (some of which are known to cause nasal cavity tumours in rodents) are associated with this tumour induction is not established. Concentrations of volatile nitrosamines in foods consumed by inhabitants of areas where there is a high risk for nasopharyngeal cancer (Tunisia, Southern China, Greenland) were found to be no higher than those in foods consumed in low-incidence areas (Japan and western Europe) (p. 415).

A few papers report attempts to assess occupational exposure to NOC. Mononitroso-piperazine was detected in the urine of factory workers engaged in the manufacture of piperazine (p. 553). Exposure of metal grinders to NDELA has been studied by monitoring urinary excretion of the unmetabolized compounds; exposure occurs mainly through the skin, and clearance from body is slow (p. 550).

The papers presented at the meeting report a number of recently developed sensitive methods for measuring biologically effective doses of NOC; their application in human studies would be a most promising future approach. Incorporation of such dosimetry studies in clinical and epidemiological investigations should lead to the timely identification of subjects and populations at high risk, due to a higher burden of nitroso compounds from endogenous and exogenous sources, and thus indicates the possibility of taking preventive measures.

**KEYNOTE ADDRESS:**  
**THOUGHTS ON NITROSAMINES AND THE CAUSE  
AND PREVENTION OF HUMAN CANCER**

**P.N. Magee**

*Fels Research Institute and Department of Pathology,  
Temple University, Philadelphia, PA 19140, USA*

Interest in the carcinogenic and mutagenic *N*-nitroso compounds has grown rapidly in recent years, as pointed out by Preussmann and Stewart in their recent review (Preussmann & Stewart, 1984), who state that, at their time of writing, about three papers were being published each day on this topic in the scientific literature. Further evidence is provided by the increasing size of the books containing the proceedings of these IARC meetings on *N*-nitroso compounds, culminating in the 1011-page volume reporting the Banff meeting of 1983. The titles of these meetings are instructive because they reflect a progressive movement away from emphasis on formation and analysis of the compounds in the environment to the more recent introduction of biological effects in Tokyo in 1981, through 'Occurrence, Biological Effects and Relevance to Human Cancer' in Banff to the present title, which is, of course, simply 'Relevance of *N*-Nitroso Compounds to Human Cancer. Exposure and Mechanisms.' This progression reflects the increasingly widely held view, contrary to that of John Barnes in 1974, that perhaps nitrosamines really are involved in the causation of some human cancer, and leads to the corollary that research aimed at the prevention of nitrosamine-induced cancers may be urgently needed. In this presentation, the metabolism and biological activation of the nitrosamines and their implications for the possible causation and prevention of human cancer are very briefly discussed.

There is substantial evidence that several nitrosamines undergo metabolism to yield reactive alkylating intermediates, increasing evidence that metabolic denitrosation may occur and some evidence for a reductive pathway leading to the alkyl hydrazine (Preussmann & Stewart, 1984). The prevailing current view is that the carcinogenic and mutagenic actions of the compounds are mediated *via* the alkylation pathway, whereas denitrosation and reduction are thought to be deactivating mechanisms leading to less toxic or nontoxic products. The obvious implication is that initiation of cancer might be prevented if ways could be found to shift the balance of metabolism away from the alkylating pathway and toward the others — notably, denitrosation.

It is widely stated that the mutagenic actions of the nitroso compounds result from their capacity to interact covalently with DNA *in vivo*; their carcinogenicity and cytotoxicity might also be due to this interaction, although reaction with proteins and other cellular constituents may be involved in the latter activities. These views may be insufficiently critical, however, since several nitroso compounds appear not to alkylate DNA but are powerfully carcinogenic. Among these are *N*-nitrosophenylmethylamine, *N*-nitrosomethylaniline (Michejda, 1986) and *N*-nitrosodiethanolamine (Lijinsky & Kovatch, 1985),

which were found to react with DNA to an extremely small extent, if at all. Regardless of its biological significance, however, the presence of detectable alkylation of nucleic acids has provided convincing evidence of exposure of human subjects, as in the demonstration of *N*7-methylguanine and *O*6-methylguanine in the liver DNA of a victim of homicidal poisoning by *N*-nitrosodimethylamine (NDMA; Herron & Shank, 1980). The much smaller levels of *O*6-methylguanine found in the oesophageal DNA of individuals living in areas of high incidence of tumours of this organ in China (Umbenhauer *et al.*, 1985) may also indicate exposure.

The evidence that nitrosamines can cause human cancer, although circumstantial, is strong and has been extensively discussed (Magee, 1982; Craddock, 1983; Bartsch & Montesano, 1984). Whether the compounds actually have and do cause the disease in human beings is much more problematical and is proving very difficult to establish one way or the other. Exposure may be to preformed nitrosamines in the environment, extensively reviewed and discussed (Preussmann, 1983; Preussmann & Eisenbrand, 1984), and/or to the products of endogenous nitrosation. The occurrence of the latter reaction in humans was clearly demonstrated by Ohshima and Bartsch in 1981, and their *N*-nitrosoproline excretion test is being widely used in conjunction with epidemiological studies throughout the world.

#### **NDMA in circulating human blood**

Very low concentrations of NDMA were found in the blood of human subjects after consumption of a meal rich in nitrate/nitrite by Fine and his colleagues in 1977, and similar findings have been reported subsequently by other workers, with or without prior raised nitrate/nitrite intake. Some investigators, however, failed to detect any nitrosamines in human blood. On the basis of analyses of more than 200 samples, Dunn and his associates (1986) concluded that low levels (range, 0.05-1.9  $\mu\text{g}/\text{kg}$ ) of NDMA can occur in human beings; but they did not detect any other nitrosamine.

In view of the greatly predominant role of the liver in the metabolism of NDMA in rats (Diaz Gomez *et al.*, 1977), and the assumption that conditions are similar in humans, it is difficult to explain how detectable circulating levels could arise from endogenous nitrosation in the human gastrointestinal tract. Recent pharmacokinetic studies of NDMA in dogs (Gombar *et al.*, 1987) and pigs (Gombar, personal communication), using the model reported at this meeting by Magee *et al.*, have shown that the hepatic extraction in these species is considerably less than in rats. The oral bioavailability of NDMA, i.e., the fraction of an orally administered dose that reaches the systemic circulation, is substantially greater in dogs and pigs than in rats. If humans resemble these larger animals more closely than rodents in this regard, the presence of circulating NDMA may be more easily explicable. As shown by Swann and his colleagues (1984), hepatic metabolism of NDMA in rabbits is profoundly inhibited by ethanol, which greatly reduces the hepatic extraction of the carcinogen and results in greater exposure of extrahepatic organs. Extraction of NDMA by the liver *in vivo* is also markedly inhibited by alcohol in pigs (unpublished results). Swann has suggested that such a mechanism may explain the increased incidence of human cancer associated with excessive consumption of alcohol. Obviously, other inhibitors of hepatic metabolism, perhaps occurring naturally, could similarly increase the exposure of extrahepatic organs to nitrosamines absorbed from the gastrointestinal tract.

**Prevention of nitrosamine-induced cancer**

The presence of various amines in some foods, notably fish (Kawamura *et al.*, 1971a,b; Zeisel & DaCosta, 1986), and nitrate/nitrite in some vegetables and drinking-water indicates that some carcinogenic nitrosamines must be formed endogenously, in addition to the preformed nitrosamines that may occur in various foods (Preussmann & Eisenbrand, 1984). Whether the extent of exposure to these carcinogens is sufficient to result in human cancer is not known. However, it should be remembered that Druckrey *et al.* (1967) showed that the product of the daily dose of carcinogen multiplied by the induction time in days raised to an exponent in the range 1.2 to 4.0 is constant in several experimental models. Since human beings may be exposed to NDMA and other *N*-nitroso compounds throughout their lives, even very low levels of exposure may be significant.

If the possibility is entertained that nitrosamines contribute to the total burden of human cancer, preventive measures are clearly indicated. An obvious way to reduce total exposure is to remove or greatly reduce exogenous sources of the compounds, including the inhibition of gastric nitrosation by vitamins C and E. Once the nitrosamine has been absorbed, its carcinogenic effect could, in theory, be reduced by selective inhibition of its metabolism to the active alkylating intermediate in favour of the denitrosation or deactivating pathway. Another possibility would be to trap the active species by reaction with receptor molecules and thus deflect it from the crucial intracellular target, DNA. Although theoretically possible, both of these preventive measures would be very difficult to achieve *in vivo*. Finally, once cancer initiation has taken place it seems probable that the subsequent preventive procedures would be independent of the chemical nature of the initiation; experiments in rodents have shown that retinoids, selenium, indomethacin, dehydro-epiandrosterone and other inhibitors influence promotion and progression after initiation by nitroso compounds in much the same way as with other carcinogens.

**Acknowledgements**

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# **MOLECULAR AND BIOCHEMICAL MECHANISMS**

# BIOCHEMICAL EVENTS IN NITROSAMINE-INDUCED HEPATOCARCINOGENESIS: RELEVANCE OF ANIMAL DATA TO HUMAN CARCINOGENESIS

R. Schulte-Hermann

*Institut für Tumorbologie, Krebsforschung der Universität Wien,  
Vienna, Austria*

**Administration of carcinogens to experimental animals results in the early formation of phenotypically altered cell foci. Evidence suggesting their intermediary role in hepatocarcinogenesis, and some of their biological properties are reviewed. Two concepts relating to the mechanism of tumour promotion are described briefly, i.e., the 'resistant cell' hypothesis and that of 'overexpression of adaptive growth'. It is concluded that studies on liver foci may be helpful in elucidating the nature of the events involved in the initiation of carcinogenesis. Some aspects relating to human hepatocarcinogenesis are also considered briefly.**

Liver carcinogenesis by nitrosamines and other carcinogens is a slow, multistep process. In this paper, some biochemical and biological aspects of this process are reviewed.

The first step includes absorption, distribution and metabolism of nitrosamines. Electrophilic metabolites that are formed bind covalently to cellular constituents, thereby causing various types of damage, such as mutations and cell death. Apparently, cancer cells are not formed immediately. Rather, the genotoxic activity of the nitrosamines appears to result in a change, termed 'initiation', which constitutes the second step of the whole process. Initiation affects only a few cells in the liver ('initiated cells'), and these multiply and undergo several further stepwise changes ('progression'), until the first malignant cell is produced. Initiated cells appear to have a proliferation advantage over normal cells under certain environmental conditions ('tumour promotion'; Boutwell, 1974; Hecker, 1978; Farber & Cameron, 1980; Pitot & Sirica, 1980; Schulte-Hermann, 1985).

Studies have already largely clarified the first step of chemical carcinogenesis, i.e., the formation and immediate effects of electrophilic metabolites. The challenge now is to elucidate the next step — initiation. What is the molecular basis of initiation, and what are its biological consequences for the cell?

In order to answer these questions, forward and backward strategies may be used, and the results may be combined. The forward strategy follows the fate of the carcinogens, studies the sites and types of binding within the cell, repair processes, etc, and tries to identify the individual genes (and possible other regulatory molecules), damage of which leads to initiation. The problem with this approach is the rarity of initiation. Even a high dose of a hepatotropic nitrosamine may produce only one initiated cell among  $10^6$  hepatocytes (see below). Identification of the critical lesion(s), such as mutation, translocation or amplification of a single or a few genes, in a single cell out of  $10^6$  cells against a vast background of nonspecific lesions appears not (yet) possible by a straightforward approach.

In a backward strategy, the progeny of initiated cells would be investigated. Studies at the molecular level of tumours of, e.g., the mammary gland or the lymphatic system have already yielded important information about (onco)genes that may be involved in the initiation of chemical carcinogenesis. Thus, a point mutation of the *Ha-ras* gene at codon 12 (GGA → GAA) seems to be an early event in mammary carcinogenesis initiated in rats by *N*-methyl-*N*-nitrosourea (Zarbl *et al.*, 1985). Other studies have identified translocation or amplification of the *c-myc* oncogene as an early lesion associated with initiation of carcinogenesis by chemicals (Klein & Klein, 1985). However, the immediate biological consequences of such lesions are not yet known.

Investigation of initiation at the biological level is hampered even more by the rarity of this event, and by the multitude and complexity of alterations in the end stage, i.e., the tumour. It is, therefore, desirable to study the progeny of initiated cells as soon as their number is sufficiently large. Fortunately, the livers of experimental animals offer a system in which the putative progeny of initiated hepatocytes can be identified at a very early stage of development as groups or foci of altered cells (Farber & Cameron, 1980; Pitot & Sirica, 1980; Bannasch *et al.*, 1984; Schulte-Hermann, 1985). Studies on the phenotypes of such cells and on their responses to modifiers such as tumour promoters should be helpful in elucidating phenotypic and — eventually — genotypic deviations responsible for their 'initiated' state. In the following, a brief summary of experimental observations and of current concepts is given.

- (1) Genetic analyses strongly suggest that the liver foci of altered cells are clones derived from a single cell (Rabes *et al.*, 1982).
- (2) A single dose of a hepatocarcinogenic nitrosamine to rats produces one focus (= one clone) per  $10^6$  hepatocytes in the liver (Farber & Cameron, 1980; Pitot & Sirica, 1980).
- (3) Foci are not produced by nitrosamines in the absence of cell proliferation. Thus, initiation seems to require a round of cell replication after contact with the carcinogen (Farber & Cameron, 1980).
- (4) Foci are seen only after exposure to carcinogens, at least in young experimental animals; in aged rats, 'spontaneous' foci have been noted (Schulte-Hermann *et al.*, 1983). They appear early, long before carcinomas, and they persist during the animals' lifetime. These findings strongly support their presumed preneoplastic state.
- (5) Foci exhibit much higher proliferative activity than normal liver, which is, however, largely counterbalanced by increased cell death (apoptosis) and by phenotypic 'remodelling' (Schulte-Hermann *et al.*, 1982; Bursch *et al.*, 1984). Therefore, most foci show relatively slow growth. Treatment with liver tumour promoters greatly accelerates focal growth, by one or more of the following mechanisms: increase in cell proliferation (Farber & Cameron, 1980; Schulte-Hermann *et al.*, 1982; Solt *et al.*, 1983), inhibition of remodelling (Farber & Cameron, 1980; Schulte-Hermann, 1985; Moore & Kitagawa, 1986) and, interestingly, inhibition of cell death (Bursch *et al.*, 1984). Thus, foci show a growth advantage during promotion.
- (6) Foci may exhibit a large number of phenotypic variations from normal liver (Bannasch *et al.*, 1982; Peraino *et al.*, 1983; Schulte-Hermann, 1985; Moore & Kitagawa, 1986; Reinacher *et al.*, 1986). Do these changes reflect chaos in gene alterations due to a multiplicity of mutational events, as was originally believed? And how do these changes relate to the growth advantage of foci during tumour promotion? Studies with two different experimental models have yielded the following two hypotheses:

(a) *Resistant cell hypothesis*: During exposure to cytotoxic compounds or diets, the specific phenotype of foci provides (relative) resistance of cells by increases in detoxifying

enzymes, membrane changes, etc. Thus, cells can better respond to proliferative (regenerative) stimuli (Farber & Cameron, 1980; Solt *et al.*, 1983).

(b) *Overexpression of adaptive growth*: Tumour promoters such as phenobarbital, hexachlorocyclohexane, DDT and certain sex steroids produce no significant sign of hepatotoxicity. They do produce, however, adaptive growth in the liver, i.e., growth (by cell enlargement and/or cell multiplication) associated with increases in drug-metabolizing enzymes and changes in carbohydrate metabolism. Cells in foci seem to overexpress many of these adaptive changes and therefore may show unbalanced (adaptive) growth (Schulte-Hermann *et al.*, 1984a,b, 1986; Schulte-Hermann, 1985).

We conclude from these and other findings that the phenotypic alterations in putative preneoplastic foci are not due to a multiplicity of random mutations caused during initiation in the target cells. Rather, we believe that the foci express ordered gene programmes *qualitatively* similar to those available in normal liver under conditions of toxic or functional load. Therefore, the phenotypic alterations in foci probably represent a new state of differentiation in which the cells appear to be 'over-specialized' for efficient performance of protective or adaptive functions. With respect to the nature of initiation, this would imply that the crucial lesion during initiation may affect master genes that govern expression of these hepatic functions.

With respect to human liver cancer, the following points should be mentioned:

- (1) The relevance of nitrosamines in human hepatocarcinogenesis is still uncertain. Hepatitis B virus, ethanol and aflatoxin B<sub>1</sub> have been recognized as major risk factors for the development of liver cancer. Some epidemiological studies suggest that smoking may also be a risk factor (IARC, 1986a) and, thus, nitrosamines contained in tobacco smoke may contribute to carcinogenesis in human liver. Tobacco smoke and ethanol may have additive effects, as in other organs.
- (2) Several putative early stages of cancer have been identified in human liver (Ho *et al.*, 1981). Their biology remains to be studied in greater detail before comparisons with the animal data reviewed above are possible.
- (3) Even though steroids do not exhibit detectable genotoxic activity, they may — very rarely — produce liver tumours in humans (IARC, 1979; Schuppler *et al.*, 1982). They do promote liver tumour development in animal experiments (Schuppler *et al.*, 1982; Schulte-Hermann, 1985; Moore & Kitagawa, 1986). Hypothetically, these agents could also promote tumour formation in human liver from initiated cells that may (rarely) arise due to contact with exogenous or endogenous carcinogens.
- (4) Liver foci in experimental animals may serve as an endpoint in test systems to detect tumour initiating and promoting agents and to quantify their efficiency. Therefore, these test systems may contribute to assessing and reducing human health risks from exposure to chemical compounds (Schulte-Hermann, 1985).



# BIOCHEMICAL AND MOLECULAR EFFECTS OF N-NITROSO COMPOUNDS IN HUMAN CULTURED CELLS: AN OVERVIEW

C.C. Harris

*Laboratory of Human Carcinogenesis, Division of Cancer Etiology,  
National Cancer Institute, Bethesda, MD 20892, USA*

**In-vitro models using human tissues and cells provide a bridge between studies of humans and of laboratory animals. Cultured human cells activate *N*-nitrosamines to DNA-damaging metabolites, including alkyl diazonium ions and aldehydes; these metabolites can also inactivate DNA repair processes, such as *O*<sup>6</sup>-alkylguanine DNA-alkyltransferase (AAT) activity, and are mutagenic in human cells. Both human epithelial and mesenchymal cells have been transformed *in vitro* by *N*-nitroso compounds.**

*N*-Nitroso compounds (NOC), including *N*-nitrosamines, cause cancer in more than 30 animal species, transform human cells *in vitro* and are probably carcinogens in humans (see review by Bartsch & Montesano, 1984). NOC are found in the general environment, in various occupational settings and in tobacco smoke (Table 1). Certain *N*-nitrosamines are present in higher concentrations in sidestream than in mainstream smoke, and tobacco-specific *N*-nitrosamines, i.e., nicotine derivatives, have been detected (Hoffmann & Hecht, 1985). *N*-Nitrosamines are also found in the general environment and are formed endogenously by in-vivo nitrosation of secondary amines.

This brief report reviews studies of NOC using primarily human cells and tissues *in vitro* to investigate the (i) metabolism of *N*-nitrosamines, (ii) formation of DNA adducts, (iii) repair of DNA lesions caused by metabolites of *N*-nitrosamines, (iv) induction of mutations and chromosomal aberrations in mammalian cells, (v) transformation of human cells, and (vi) mechanism(s) by which NOC cause genetic damage.

## **Metabolism of *N*-nitrosamines and induction of DNA damage**

Since many chemical carcinogens require metabolic activation before they can exert their mutagenic and carcinogenic effects, it is important to determine if human target tissues can metabolize these procarcinogens into their ultimate carcinogenic forms. Investigations of chemical carcinogenesis in human tissues have been facilitated by the progress made in the culture of human epithelial tissues and cells during the last decade (see review by Harris, 1987). Methods have been developed to culture normal tissues and cells from the major sites of human cancer. Chemically defined media have been devised both for explant culture of human bronchus, colon, oesophagus and pancreatic duct and for culture of isolated human epithelial cells from bronchus, bladder, prostate, mammary gland and skin. These in-vitro model systems provide an important bridge between epidemiology and studies using experimental animals in the investigation of carcinogenesis.

Important aspects of studies of carcinogenesis using cultured human tissues and cells include elucidation of the possible role of metabolism, DNA damage and its repair in relation to the relative organ specificity shown by many chemical carcinogens. For example, *N*-nitrosamines are potent and organotropic carcinogens in laboratory animals; following metabolic activation, they give rise to alkyl diazonium ions, nitrogen and aldehydes (Fig. 1).

**Table 1. *N*-Nitrosamines in cigarette smoke<sup>a</sup>**

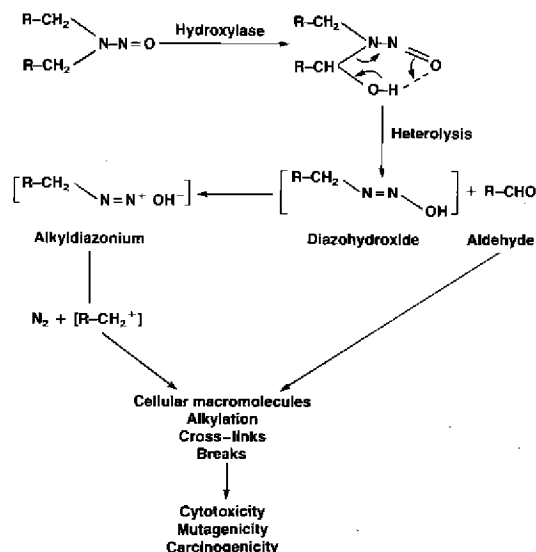
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<i>N</i> -Nitrosodimethylamine
<i>N</i> -Nitrosoethylmethylamine
<i>N</i> -Nitrosodiethylamine
<i>N</i> -Nitrosodi- <i>n</i> -propylamine
<i>N</i> -Nitrosodi- <i>n</i> -butylamine
<i>N</i> -Nitrosopyrrolidine
<i>N</i> -Nitrosopiperidine
<i>N</i> -Nitrosodiethanolamine
<i>N</i> '-Nitrosornicotine
4-(Nitrosomethylamino)-1-(3-pyridyl)- 1-butanone
<i>N</i> '-Nitrosoanatabine
<i>N</i> '-Nitrosoanabasine

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<sup>a</sup>From Hoffmann & Hecht (1985)

**Fig. 1. Metabolic activation and pathobiological effects of *N*-nitrosamines**



Metabolism of *N*-nitrosodimethylamine (NDMA) gives rise to at least two products that can react with nucleophilic sites in cellular macromolecules: methyl diazonium ions by alkylation and formaldehyde *via* formation of unstable methylol derivatives with amine groups (R-HN-CH<sub>2</sub>OH). These hydroxymethylated derivatives can, by a slow spontaneous secondary reaction, yield stable methylene bridges between macromolecules. The amounts of methyl diazonium ion and aldehyde bound to macromolecules are dependent on many physicochemical factors (Auerbach *et al.*, 1977; Hemminki, 1981). Formaldehyde may also be rapidly oxidized in the cell to formate, and ultimately to carbon dioxide, by the involvement of different enzymatic pathways. Both formaldehyde and formate may eventually enter the one-carbon pool as N<sup>5</sup>-, N<sup>10</sup>-tetrahydrofolate derivatives and subsequently become incorporated into a variety of cellular products.

Because formation of the alkyl diazonium ion is regarded as essential for initiation of *N*-nitrosamine carcinogenesis, a possible contribution of aldehydes has received less attention and study. However, formaldehyde, formed by metabolic activation of NDMA and by demethylation of a variety of other xenobiotics (Waydhas *et al.*, 1978), has been shown to be mutagenic in several species (Auerbach *et al.*, 1977), including cultured human cells (Goldmacher & Thilly, 1983; Grafstrom *et al.*, 1985), and a respiratory carcinogen in rodents (Svenberg *et al.*, 1980). Therefore, formaldehyde can be considered as a potential carcinogen in humans (Federal Panel on Formaldehyde, 1982).

Both acyclic and cyclic *N*-nitrosamines can be activated to metabolites that are associated with DNA in cultured human epithelial tissues and cells (Harris *et al.*, 1977; Autrup *et al.*, 1978; Harris *et al.*, 1979; Autrup & Stoner, 1982; Table 2). Human bronchus can activate all of the *N*-nitrosamines tested to date. Radioactivity associated with DNA was observed in human colon incubated with *N*-nitrosopyrrolidine, but no detectable radioactivity was found with *N*-nitrosopiperidine; neither of these cyclic *N*-nitrosamines was activated to metabolites associated with DNA in cultured human oesophagus, although radioactivity associated with proteins was observed. In contrast, cultured rat oesophagus can readily activate cyclic *N*-nitrosamines to metabolites associated with DNA, including the potent organotrophic carcinogen in rats — *N*-nitrosomethylbenzylamine (NMBzA; Table 3; Autrup & Stoner, 1982). A preference for metabolic  $\alpha$ -oxidation at the methyl group was indicated by ten-fold higher levels of benzaldehyde than formaldehyde and carbon dioxide. The activation of *N*-nitrosopyrrolidine was greater in human bronchus and colon than in other human tissues, but an even higher level of DNA binding was seen in the corresponding rat tissues. Both *N*<sup>7</sup>- and *O*<sup>6</sup>-methylguanine adducts were detected after incubation of human tissues with <sup>14</sup>C-NDMA.

**Table 2. Variation among three human tissues in metabolism of *N*-nitrosamines to form DNA adducts<sup>a</sup>**

<i>N</i> -Nitrosamine	Carcinogen-DNA adduct formation		
	Bronchus	Colon	Oesophagus
<i>N</i> -Nitrosodimethylamine	100	6	67
<i>N</i> -Nitrosodiethylamine	78	8	100
<i>N</i> -Nitrosopyrrolidine	100	52	ND <sup>b</sup>

<sup>a</sup>The tissue type with the highest number of DNA adducts is given the value of 100 percent. Normal tissue explants from immediate autopsy donors were exposed to 100  $\mu$ M of *N*-nitrosamine for 24 h (Harris *et al.*, 1979).

<sup>b</sup>Not detected

**Table 3. Comparison of DNA binding of *N*-nitrosamine metabolites in cultured human and rat oesophagus<sup>a</sup>**

<i>N</i> -Nitrosamine	Human:rat ratio
<i>N</i> -Nitrosodimethylamine	0.9
<i>N</i> -Nitrosodiethylamine	1.0
<i>N</i> -Nitrosomethylbenzylamine	0.012

<sup>a</sup>From Autrup & Stoner (1982)

& Montesano, 1985). AAT activity increases before the initiation of DNA synthesis in S-phase of normal human cells (Kim *et al.*, 1986) and varies widely among different human tissue types and among animal species (see reviews by Krokan *et al.*, 1986; Wild *et al.*, 1986).

Interspecies differences have been found in the metabolism of *N*-nitrosamines such as NMBzA, described above, and *N*<sup>7</sup>-nitrosornicotine, in which the 2' to 5' hydroxylation ratio is lower in human tissues than in tissues from rat, mouse or Syrian golden hamster (Table 4).

### DNA repair

Although formaldehyde-induced DNA damage in human cells appears to be repaired by an excision mechanism (Grafstrom *et al.*, 1984), several different enzymes repair alkyl-diazonium ion-induced DNA damage, e.g., *O*<sup>6</sup>-methylguanine is repaired by an AAT (Pegg, 1986; Yarosh, 1985), *N*<sup>3</sup>-methyladenine and *N*<sup>7</sup>-methylguanine are repaired by a specific DNA glycosylase (Male *et al.*, 1981), and *O*<sup>4</sup>-methylthymidine is repaired by an as yet undefined enzyme (Becker

This DNA repair enzyme has a cysteine at its active site and can be inactivated by alkylating agents (Pegg *et al.*, 1983; Brent, 1986) and by aldehydes, including those found in tobacco smoke and formed by the metabolism of *N*-nitrosamines (Fig. 1; Grafstrom *et al.*, 1985; Krokan *et al.*, 1985a, 1986). Normal human bronchial epithelial cells *in vitro* do not show an adaptive response after treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Krokan *et al.*, 1985b).

**Table 4. Relative regiospecificity among animal species and tissues in the metabolism of *N'*-nitrosornicotine (NNN)<sup>a</sup>**

Species and tissue	<i>N</i> -Oxidation (%)	2':5' Hydroxylation ratio
Fischer 344 rat oesophagus	0.9	3.0
Syrian golden hamster oesophagus	3.6	0.3
Human oesophagus	0.3	< 0.1 <sup>b</sup>
A/J mouse lung	3.2	0.6
Human lung	0.6	< 0.1 <sup>b</sup>
Human bronchus	0.2	< 0.1 <sup>b</sup>

<sup>a</sup>Tissue explants were cultured with [<sup>2-14</sup>C]-NNN for 24 h.

<sup>b</sup>2'-Hydroxylation was not detected; conversion of NNN to metabolites (up to 0.8%) was lower in human tissues than in those from laboratory animals (30-60%; Hecht *et al.*, 1982).

Recent data suggest that AAT may have selectivity for different base sequences (Topal *et al.*, 1986). As shown in Table 5, a DNA sequence surrounding a guanine site that is poorly repaired has been derived by consensus. This sequence has a significant similarity to the region of the rat *Ha-ras* oncogene containing the *N*-methyl-*N*-nitrosourea (MNU)-activated codon 12 site for neoplastic transformation (Zarbl *et al.*, 1985). Topal *et al.* (1986) propose that direct alkylation at the O<sup>6</sup> position of guanine present within the consensus sequence produces a DNA conformation less subject to repair by AAT.

**Table 5. O<sup>6</sup>-Methylguanine mutation and repair is nonuniform<sup>a</sup>**

	-20		0		+20
Human <i>H-ras</i>	ctggtggt	GgTGGgCGC C-	GGc	GG	TgtggGCaagagtCCgo
Rat <i>H-ras</i>	cttgtggt	GgTGGgCGCt-	GGA	GG	cgtggGaaagagtGCcc
Consensus	AGCACCAG	GCTGGTGGC CA	G*A	GC	TCATCGCGCCTAGGCTG
			codon 12		
			similarity = 12/16 (75%)		

<sup>a</sup>From Topal *et al.* (1986)

### Mutagenesis

Cell-mediated mutagenesis assays have further extended our understanding of the interspecies differences found in the metabolism of *N*-nitrosamines, described above. As shown in Table 6, rat oesophageal epithelial cells activate NMBzA more efficiently than do either chicken or human oesophageal cells (Cheng & Li, 1985). These results suggest that NMBzA would be only weakly potent in inducing tumours in chicken and human oesophagus; a recent study showed this *N*-nitrosamine to be ineffective in causing oesophageal carcinomas in chickens (Li, M. *et al.*, 1985).

**Table 6. Induction of 6-thioguanine resistant (6-TG) mutants by NMBzA in Chinese hamster V79 cells cocultivated with oesophageal epithelial cells from rats, chickens and humans<sup>a</sup>**

Group	6-TG Mutants/10 <sup>6</sup> survivors <sup>b</sup>
V79 cells alone	7 (1-21)
V79 cells plus:	
Rat oesophageal cells	144 (87-193)
Chicken oesophageal cells	10 (8-11)
Human oesophageal cells	12 (1-33)

<sup>a</sup>From Cheng & Li (1985); 10<sup>-3</sup> M NMBzA, 24 h

<sup>b</sup>Mean (range)

frequency of induction of 6-thioguanine-resistant mutants in human fibroblasts after their exposure to the compounds (Fig. 2). Although the exposure times differed, formaldehyde was three times more mutagenic than MNU on a molar basis. MNU and formaldehyde were each weak mutagens at the concentrations tested; however, addition of 50 or 75  $\mu$ M formaldehyde to 200  $\mu$ M MNU-treated cells resulted in a mutation frequency that was significantly greater than that found with either agent alone.

Formaldehyde may increase the mutagenicity of MNU by inhibiting *O*<sup>6</sup>-methylguanine repair. This hypothesis is strengthened by analysing the frequency of 6-thioguanine-resistant mutants as a function of the logarithm of the percentage of survival. The curves for MNU and for formaldehyde are of similar magnitude and shape (Grafstrom *et al.*, 1985); however, a much steeper curve is found when formaldehyde and MNU are combined under conditions known to inhibit the repair of *O*<sup>6</sup>-methylguanine. This indicates that the combination of MNU and formaldehyde is more mutagenic than either agent alone. This is to be expected if the persistence of the *O*<sup>6</sup>-methylguanine lesion has a more significant effect on induced mutation than it does on cell killing. Thus, there is a good correlation between the inhibition of *O*<sup>6</sup>-methylguanine removal and the synergistic increase in mutation frequency in the presence of formaldehyde. This is also further indirect evidence that *O*<sup>6</sup>-methylguanine is a promutagenic DNA lesion. In addition, MNU may inhibit the repair of promutagenic lesions caused by formaldehyde.

### Neoplastic transformation

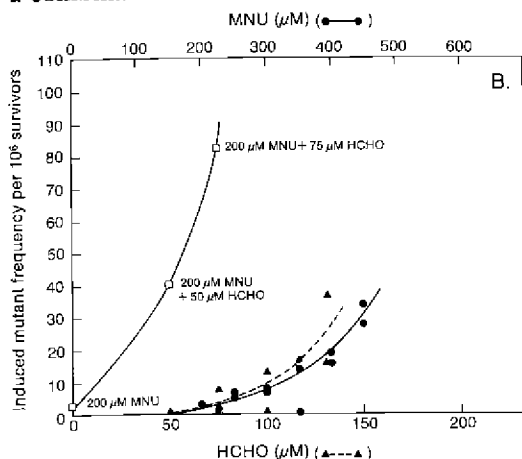
In-vitro transformation of normal human epithelial, lymphoid and mesenchymal cells to malignancy has proven difficult but has recently been achieved (see review by Harris, 1987). Human cells from several types of tissues have been either partially or completely transformed by NOC to malignant cells that form progressively growing tumours in athymic nude mice (Table 7).

### Mechanism of genetic toxicity

*N*-Nitrosamines can be metabolized by cultured human epithelial tissues and cells. Quantitative differences in and alkylation of DNA are found among humans and among different organs within an individual. Whether or not these differences are sufficient to influence an individual's cancer risk and organ site is as yet unknown.

To study further the pathobiological consequences of exposure to formaldehyde and inhibition of *O*<sup>6</sup>-methylguanine repair, we investigated the cytotoxic and mutagenic effects of formaldehyde and MNU separately and in combination (Grafstrom *et al.*, 1985). Cytotoxicity was determined by measuring the colony-forming efficiency of human fibroblasts that had been exposed to 100-800  $\mu$ M MNU for 1 h or 50-175  $\mu$ M formaldehyde for 5 h, or to both. The effect of low concentrations of formaldehyde or MNU or both was assessed by measuring the fre-

**Fig. 2. Frequency of 6-thioguanine-resistant mutants induced as a consequence of treatment with MNU, formaldehyde (HCHO), or a combination of the two**



mutations (Zarbl *et al.*, 1985) at guanine sites within consensus DNA sequences that may be sterically hindered to repair by AAT (Topal *et al.*, 1986). In addition to point mutations, NOC cause DNA single-strand breaks, chromosomal deletions and rearrangements (Waldren *et al.*, 1986), which may also contribute to their genotoxicity. These nonpoint mutations are also caused by aldehydes.

While the alkylating metabolites of *N*-nitrosamines and their cytotoxic, mutagenic and carcinogenic effects have been extensively studied, the possible contribution of other metabolites, especially aldehydes, has not received much attention. Results from studies using experimental animals and our results showing multiple effects (DNA-protein cross-links, DNA single-strand breaks, inhibition of DNA repair and cytotoxicity) of formaldehyde in cultured human bronchial cells suggest that the major metabolites of *N*-nitrosamines, i.e., alkyl-diazonium ions and aldehydes, may act in concert to produce the toxic, mutagenic and carcinogenic effects of *N*-nitrosamines.

NOC can activate *Ha-ras* oncogene in laboratory animals by causing point

**Table 7. In-vitro transformation of human cells by *N*-nitroso compounds<sup>a</sup>**

Tissue type	Agents	Extended in-vitro life span	Immortalization	Karyotype	Transformation assay		
					Anchorage-independent growth <i>in vitro</i>	Progressively growing subcutaneous tumours in reviews thymic nude mice	Examples of reports and
Skin	SV-40 adeno-12 plus MNNG	+	+	A	+	+	Rhim <i>et al.</i> (1986)
	MNNG	+	-	NR	+	-	Milo & DiPaolo (1978)
Pancreas	MNU	+	+	NR	NR	+	Parsa <i>et al.</i> (1981)
Oesophagus	NDEA	+	-	A	+	-	Huang <i>et al.</i> (1986)
Lung	NDEA	+	-	D	+	-	Emura <i>et al.</i> (1985)
Endometrium	MNNG	+	-	NR	+	-	Dorman <i>et al.</i> (1983)

<sup>a</sup>MNNG, *N*-methyl-*N'*-nitrosoguanidine; MNU, *N*-methyl-*N*-nitrosoourea; NDEA, *N*-nitrosodiethylamine; A, aneuploid; NR, not reported; D, diploid; +, positive effect; -, no observable effect

# ACTIVATION OF THE HUMAN *c-Ha-ras-1* PROTO-ONCOGENE BY IN-VITRO REACTION WITH *N*-NITROSOMETHYL(ACETOXYMETHYL)AMINE

S. Hirani-Hojatti, J.R. Milligan & M.C. Archer<sup>1</sup>

*Department of Medical Biophysics, University of Toronto, Ontario Cancer  
Institute, 500 Sherbourne Street, Toronto, Ontario M4X 1K9, Canada*

**Reaction of *N*-nitrosomethyl(acetoxymethyl)amine *in vitro* in the presence of esterase with a plasmid containing the human *c-Ha-ras-1* gene has been shown to generate a transforming oncogene when the methylated DNA is transfected into NIH 3T3 cells. Our results show that an activated nitrosamine can mutate a normal cellular proto-oncogene, a reaction that may be the necessary first step in the multistage process of tumour induction.**

A number of human tumours and malignant tumours produced in rodents by administration of chemical carcinogens contain activated *ras* oncogenes (Barbacid, 1986). For example, a high percentage of rat mammary tumours induced by a single dose of *N*-methyl-*N*-nitrosourea (MNU) contain transforming *Ha-ras-1* genes (Sukumar *et al.*, 1983; Zarbl *et al.*, 1985). Each of these *Ha-ras-1* oncogenes is activated by the same G to A transition at guanine 35 in the 12th codon. This transition was not present in *Ha-ras-1* oncogenes in 7,12-dimethylbenz[*a*]anthracene-induced breast tumours, suggesting that the G<sup>35</sup> to A mutation induced by MNU is not simply the result of a positive growth selection process, or a unique DNA repair system present in mammary cells. Furthermore, because MNU is chemically highly labile, the mutagenic effect causing transformation must occur shortly after its administration to rats. These results provide circumstantial evidence that activation of *Ha-ras-1* by MNU occurs concomitantly with the initiation of the carcinogenic process.

In the case of the polycyclic aromatic hydrocarbons and aromatic amines, there is more direct evidence that these compounds initiate carcinogenesis by interacting with a proto-oncogene. pEC is a plasmid containing a 6.6-kb Bam HI restriction fragment of the human *c-Ha-ras-1* proto-oncogene, containing its entire coding sequence, inserted into the 4.3-kb vector pBR322 (Fig. 1). Vousden *et al.* (1986) showed that reaction of pEC *in vitro* with benzo[*a*]pyrene diol epoxide or *N*-acetoxy-2-acetylaminofluorene generates a transforming oncogene when the modified DNA is introduced into NIH 3T3 cells. Such direct evidence for interaction of a proto-oncogene with simple alkylating carcinogens to produce a transforming oncogene is lacking. Here, we show that *N*-nitrosomethyl(acetoxymethyl)amine (NMAcMA), which forms the methylating agent  $\alpha$ -hydroxynitrosodimethylamine in the presence of esterase, reacts with the human *c-Ha-ras-1* gene *in vitro* to generate a transforming oncogene when the modified DNA is subsequently transfected into NIH 3T3 cells.

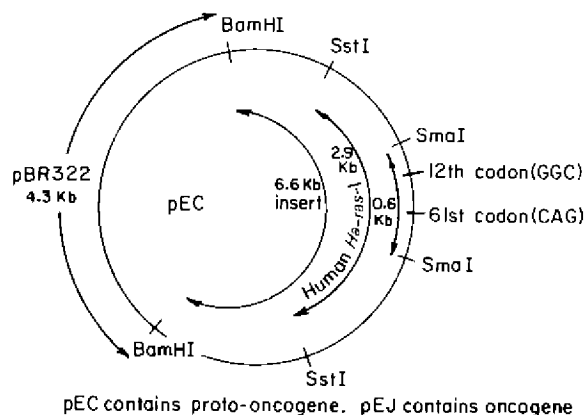
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<sup>1</sup>To whom correspondence should be addressed

### Methylation of plasmid DNA

Preliminary experiments using the plasmid pEJ, which contains the human *c-Ha-ras-1* oncogene inserted into pBR322, showed that concentrations of nitrosamine above about 10 mM produce significant reductions in transforming activity. We therefore examined the

**Fig. 1. Schematic representation of plasmids pEC and pEJ used in the methylation-transfection experiments**



**Table 1. Induction of focus-forming activity by reaction of NMAcMA with pEC plasmid**

[NMAcMA] (mM)	mmol <i>N</i> -methyl- guanine per mol guanine	mmol <i>O</i> <sup>6</sup> -methyl- guanine per mol guanine	Foci per μg DNA
0	-	-	0
0.001	0.057	0.0067	1.9
0.01	0.57	0.067	4.9
0.05	2.1	0.36	1.9
0.1	5.7	0.67	4.9
0.5	19.7	3.3	3.4
1.0	29.0	5.1	2.8
5.0	127	20.9	2.8
10.0	145	19.5	3.3

Plasmid DNA (10 μg pEC in 100 μl 10 mM Tris pH 8.0/1 mM EDTA) was treated with NMAcMA in the presence of hog liver esterase at 25°C for 16 h. The reaction mixture was then extracted with phenol, phenol:chloroform (1:1) and chloroform:isoamylalcohol (24:1). High-molecular-weight, normal mouse liver DNA (100 μg) was added, and the DNA precipitated with ethanol. Aliquots of dissolved DNA containing 1 μg plasmid DNA and 20 μg carrier DNA were co-precipitated with calcium phosphate and used to treat NIH 3T3 cells. Foci were scored after 17-18 days. In separate experiments, the amounts of *N*-methylguanine and *O*<sup>6</sup>-methylguanine formed in the plasmid DNA following reaction with NMAcMA were determined by acid hydrolysis of the DNA followed by analysis by high-performance liquid chromatography.

reaction of pEC with NMAcMA at a number of concentrations below 10 mM. Table 1 shows that the methylated plasmid DNA was able to produce foci of transformed cells at all concentrations of NMAcMA used. The number of foci produced did not change substantially, despite a variation of greater than 2000-fold in the amount of methylated guanine formed. No focus was observed in any of the plates transfected with the unreacted DNA.

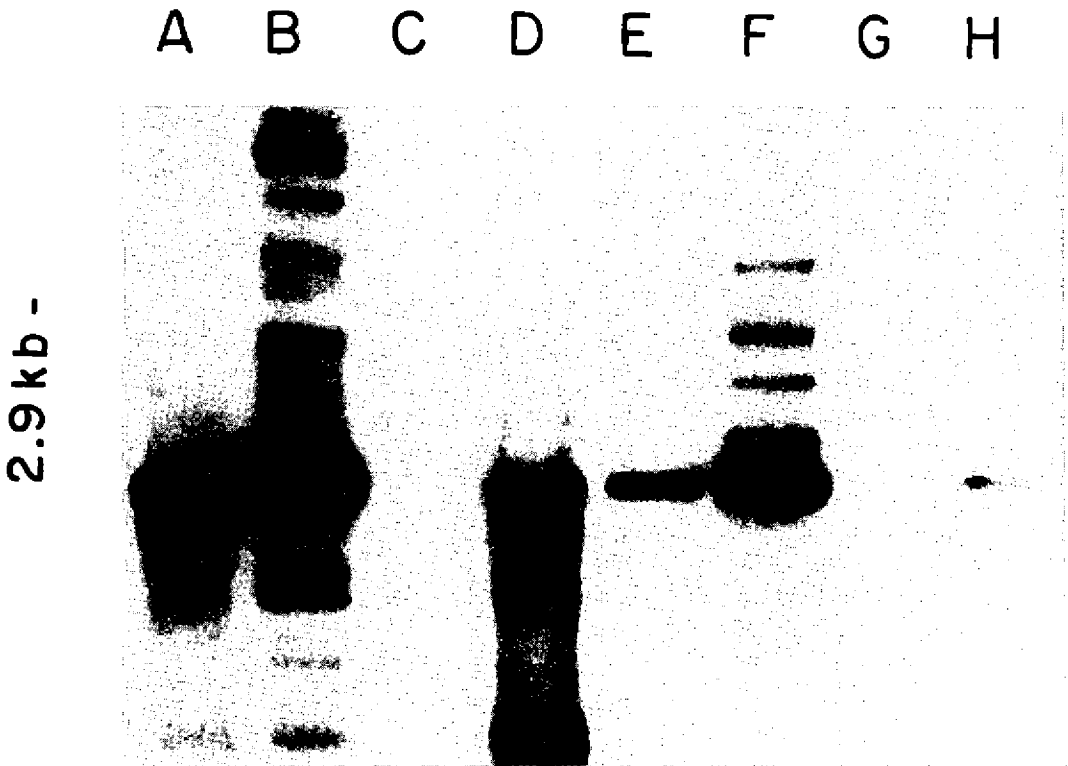
### Southern analysis of foci

To demonstrate that foci contained the transfected genes, individual foci chosen at random were trypsinized and the cells were cultured. DNA prepared from these cultures was then subjected to Southern blot analysis using a probe specific for the *c-Ha-ras-1* gene. Figure 2 shows that five out of six foci tested contained sequences derived from the transfected plasmid DNA. Focus 3 (lane C) was a spontaneous focus. It is clear from Figure 2 that there was no cross-hybridization of the probe to endogenous NIH 3T3 *c-Ha-ras-1* sequences (lane G). DNA prepared from a pEJ focus was the positive control (lane H). We have to date identified a total of nine foci that contain the transfected gene.

The intensity of the 2.9-kb fragments in the transformed foci indicates that a number of copies of the *ras* gene were integrated into the genome of the fibroblasts. However, only one, or at most a



**Fig. 2. Southern blot showing the presence of human *c-Ha-ras-1* sequences in SstI-digested DNA from primary foci produced by transfection of NIH 3T3 cells with pEC treated with NMAcMA**



Lane A, focus 1; lane B, focus 2; lane C, spontaneous focus; lane D, focus 3; lane E, focus 4; lane F, focus 5; lane G, normal NIH 3T3 cell DNA; lane H, pEJ focus. Genomic DNA ( $5 \mu\text{g}$ ) was digested with SstI, electrophoresed on a 0.8% agarose gel, and transferred to nitrocellulose. The filter was hybridized at  $42^\circ\text{C}$  in  $5 \times \text{SSC}$  buffer mixture, 50% formamide, 20 mM sodium phosphate,  $1 \times$  Denhardt's solution,  $50 \mu\text{g}/\text{ml}$  denatured salmon-sperm DNA, 10% dextran sulfate with  $2 \times 10^7$  cpm nick-translated  $^{32}\text{P}$ -labelled probe of the 2.9-kb fragment of pEC. The filter was washed in  $0.1 \times \text{SSC}/0.1\%$  sodium dodecyl sulfate at  $60^\circ\text{C}$  and autoradiographed at  $-70^\circ\text{C}$ .

few, of these copies are likely to be activated *ras* genes. The other copies will be unaltered genes or genes mutated at sites that do not lead to activation. It is unlikely that the transformants we produced are caused simply by integration of multiple copies of the unactivated *ras* gene. Although such a process has been shown to produce transformation (Pulciani *et al.*, 1985), sufficient copies are integrated into the NIH 3T3 genomes ( $>30$  copies) only when amounts of DNA  $>10 \mu\text{g}$  are used for transfection. We used one-tenth this amount of DNA. In order to eliminate these possibilities, however, we are currently making secondary transformants using DNA prepared from cells grown up from the primary foci. DNA taken up adventitiously in the first round of transfection will be lost in this process.

### Production of tumours in mice

We have tested the activity of cells grown up from the primary foci to produce tumours in mice. Male CBA/CaJ mice underwent thymectomy at four to five weeks of age. After six weeks, they were treated intraperitoneally with 1- $\beta$ -D-arabinofuranosyl cytosine (200 mg/kg), and 48 h later were given whole-body irradiation (850 rad). After a further 40 h, they were injected intraperitoneally with heterologous antilymphocyte serum (0.2 ml), and, 2 h later,  $10^6$  cells were injected subcutaneously into their flanks. Nine primary foci identified by Southern blot analysis were tested (four mice/group). Normal NIH 3T3 cells were also tested, as a negative control. Mice were given a second treatment with antilymphocyte serum 72 h after the injection with cells; 15 days later they were checked for tumour growth by palpation.

There was no tumour in the mice injected with normal NIH 3T3 cells, but cells from six of the nine foci produced tumours in all four mice, two foci produced tumours in two of four mice, and one focus gave no tumour (tumours were  $>1$  cm in diameter). We are currently examining these tumours histologically.

DNA from the tumours, and from the secondary transformants produced from both the tumour DNA and directly from transfection of the DNA from primary foci, will be subjected to Southern blot analysis to check for the presence of *c-Ha-ras-1* sequences. This DNA will also be used in restriction-fragment-length polymorphism assays to determine whether activation has resulted from point mutations at either the 12th or 61st codons. These are known sites of activation of the *ras* oncogenes in human and animal tumours (Barbacid, 1986). If other loci are involved, we shall determine the sequence of the oncogenes to locate the site of mutation.

Our results to date show that a nitrosamine that occurs in the environment, *N*-nitrosodimethylamine, can activate a human proto-oncogene. Furthermore, the results are consistent with the concept that nitrosamines could be human carcinogens. They support the concept that mutation of normal cellular proto-oncogenes by an activated carcinogen that is a simple methylating agent is the necessary first stage in the multistage process of tumour induction.

### Acknowledgements

This work was supported by the Ontario Cancer Treatment and Research Foundation and the National Cancer Institute of Canada.

## SPECIFICITY OF *O*<sup>6</sup>-ALKYLGUANINE-DNA ALKYLTRANSFERASE

A.E. Pegg, D. Scicchitano, K. Morimoto & M.E. Dolan

*Department of Physiology and Cancer Center, Milton S. Hershey Medical Center,  
The Pennsylvania State University, Hershey, PA 17033, USA*

Extensive investigations of the specificity of *O*<sup>6</sup>-alkylguanine-DNA alkyltransferase (AAT) have been carried out. These studies have shown that: (i) the mammalian protein differs from that of *Escherichia coli* in lacking the ability to remove methyl groups from *O*<sup>4</sup>-methylthymine; (ii) the protein can remove longer alkyl groups from the *O*<sup>6</sup> position but the rate of repair declines as the chain length increases; (iii) *O*<sup>6</sup>-methylguanine in RNA is much less active as a substrate for the protein than *O*<sup>6</sup>-methylguanine in double-stranded DNA; (iv) the free-base *O*<sup>6</sup>-alkylguanine is a very weak substrate for the protein so that reaction with it leads to the loss of alkyltransferase activity. (This property can be used to deplete AAT in cultured cells and in tissues and tumours after administration of *O*<sup>6</sup>-methylguanine); and (v) oligodeoxynucleotides containing *O*<sup>6</sup>-methylguanine are substrates for AAT. Such oligodeoxynucleotides can be labelled with <sup>32</sup>P at very high specific activity and can be used in an ultrasensitive assay for AAT activity.

Many *N*-nitroso carcinogens are known to be converted to alkylating intermediates which react with DNA. Several of the DNA adducts that can be formed in this reaction may have the potential to initiate mutations and tumours (Singer & Kusmierek, 1982; Singer, 1984), but *O*<sup>6</sup>-alkylguanine is thought to be the major adduct which leads to these changes, unless it is repaired prior to the onset of cell division (Pegg, 1983, 1984; Saffhill *et al.*, 1985). This repair is carried out by the protein AAT, which acts stoichiometrically to transfer the alkyl group from the DNA to a cysteine acceptor site contained within the alkyltransferase protein sequence. The ability of AAT to repair DNA damaged by alkylating agents and the cellular content of the AAT may, therefore, have an important bearing on the potential risk associated with exposure to these agents, which include metabolites from a large number of *N*-nitroso compounds. Previous studies on the cellular distribution of the AAT protein have been summarized and reviewed by Pegg (1982, 1986) and by Yarosh (1985). We have now carried out detailed investigations on the specificity of this AAT protein, which are summarized below.

### Repair of larger alkyl groups

Alkylated DNA substrates containing *O*<sup>6</sup>-alkylguanine adducts of varying sizes were prepared by reaction of calf thymus DNA with the appropriate *N*-alkyl-*N*-nitrosourea. The abilities of human, rat and *Escherichia coli* AAT to remove these alkyl groups were then determined, and it was found that larger alkyl groups could be removed from the *O*<sup>6</sup> position of guanine by these proteins; the rate of removal decreased with the size of the adduct, and branched-chain adducts were removed much more slowly than linear alkyl

groups. 2-Hydroxyethyl groups were also removed by the AAT, but at a relatively slow rate. The rates of repair of alkyl groups by mammalian AAT were in the order methyl > ethyl, *n*-propyl > *n*-butyl >> *iso*-propyl, *iso*-butyl, 2-hydroxyethyl. Results with *E. coli* AAT were qualitatively similar, but all of the adducts larger than methyl were less active substrates, ethyl and *n*-propyl groups being removed at least 100 times more slowly than methyl. This contrasts with the results for rat and human AAT, which removed ethyl and *n*-propyl groups only three to four times more slowly than they removed methyl (Pegg *et al.*, 1984; Morimoto *et al.*, 1985).

#### Repair of *O*<sup>4</sup>-methylthymine in DNA

A second major difference between the *E. coli* and mammalian AATs was revealed when the proteins were tested for their ability to remove methyl groups from the *O*<sup>4</sup> position of thymine. Substrates containing *O*<sup>4</sup>-methylthymine were prepared by the reaction of either poly d(T) or calf thymus DNA with *N*-[<sup>3</sup>H-methyl]-*N*-nitroso-urea. When these substrates were tested with the *E. coli* AAT it was found that methyl groups were removed from *O*<sup>4</sup>-methylthymine, confirming the results of McCarthy *et al.* (1984); however, no loss of *O*<sup>4</sup>-methylthymine occurred with the mammalian AATs, indicating that the latter do not remove methyl groups from the *O*<sup>4</sup> position of thymine (Dolan *et al.*, 1984; Dolan & Pegg, 1985). Therefore, in tissues that have significant AAT activity, the rapid repair of *O*<sup>6</sup>-alkylguanine along with the very slow removal of *O*<sup>4</sup>-alkylthymine may lead to a significant build-up of the relatively minor thymine adduct. This adduct might, therefore, become the important lesion in the initiation of tumours (Singer, 1984; Swenberg *et al.*, 1984; Richardson *et al.*, 1985).

#### Repair of *O*<sup>6</sup>-methylguanine in RNA

Studies using as substrates both rRNA (Pegg *et al.*, 1983) and tRNA (Table 1), alkylated by reaction with *N*-[<sup>3</sup>H-methyl]-*N*-nitroso-urea, indicated no significant removal of *O*<sup>6</sup>-methylguanine in a period sufficient for complete repair of an equivalent amount of *O*<sup>6</sup>-methylguanine in double-stranded DNA. Studies in which a large excess of AAT was added to the putative alkylated tRNA substrate indicated that the rate of repair of this base in tRNA was at least 1000 times slower than in DNA (Table 1, Experiment B). This result, which was obtained with both mammalian and *E. coli* AAT (Table 1, Experiment C), is in direct contradiction to the report published by Karran (1985). We have no explanation for this discrepancy. Our results show clearly that *O*<sup>6</sup>-methylguanine in RNA is unlikely to be a physiologically relevant substrate for the AAT in cells exposed to alkylating agents. Therefore, the possible depletion of AAT activity by reaction with RNA which would render it unable to deal with DNA lesions is improbable. This is consistent with previous studies (reviewed by Pegg, 1983), in which it has been found that the number of molecules of *O*<sup>6</sup>-methylguanine that can be repaired rapidly from the DNA of mammalian cells after exposure to methylating agents is approximately equal to the number of molecules of AAT.

#### Reaction with *O*<sup>6</sup>-alkylguanines

When mammalian cells are grown in the presence of *O*<sup>6</sup>-methylguanine or analogues, such as *O*<sup>6</sup>-*n*-butylguanine, the content of AAT is depleted (Karran & Williams, 1985; Dolan *et al.*, 1985a; Yarosh *et al.*, 1986). This loss of activity does not require the incorporation of the base into nucleic acid (Dolan *et al.*, 1985a) and appears to be brought about by the free base acting as a substrate (albeit a very weak one) for AAT. Evidence that

**Table 1. Repair of *O*<sup>6</sup>-methylguanine in tRNA by addition of AAT**

	Amount of AAT added	pmol <i>O</i> <sup>6</sup> -methylguanine in tRNA
Experiment A	0	1.63 ± 0.07
	50 units <i>E. coli</i>	1.52 ± 0.18
Experiment B	0	0.86 ± 0.02
	1705 units <i>E. coli</i>	0.46 ± 0.08
Experiment C	0	3.67
	2.2 units <i>E. coli</i>	3.67
	11 units <i>E. coli</i>	3.60
	2.8 units human (HT29)	3.56

AAT extracts from either *E. coli* or human colon tumour cells (HT29) were incubated for 1 h at 37°C with the <sup>3</sup>H-methylated tRNA substrate (3.5 mCi/μmol) containing the amount of *O*<sup>6</sup>-methylguanine shown for each experiment. At the end of this period, the amount of *O*<sup>6</sup>-methylguanine remaining in the tRNA was determined. The units of AAT were determined using a [<sup>3</sup>H-methyl]-labelled heat-treated DNA substrate and represent the amount (pmol) of *O*<sup>6</sup>-methylguanine that can be removed by the protein from DNA. This reaction was complete within 30 min. Extracts containing AAT activity were prepared from HT29 cells and from *E. coli* as described by Scicchitano *et al.* (1986).

tivity of human fibroblasts to mutagenesis by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Domoradzki *et al.*, 1985) and an increased sensitivity of human tumour cells in culture to killing by chloroethyl nitrosoureas (Dolan *et al.*, 1985b, 1986; Yarosh *et al.*, 1986) but apparently not of Raji cells (Karran & Williams, 1985). The reason that Raji cells showed this anomalous behaviour is not clear, but it may relate to the rapid rate of new synthesis of AAT in these cells.

In order to test whether treatment with *O*<sup>6</sup>-methylguanine to deplete AAT might be of value in studies involving carcinogenesis and tumour cell therapy, we examined whether sufficient doses of *O*<sup>6</sup>-methylguanine (which is not particularly soluble in aqueous media) could be given to reduce the activity in mouse liver and in human tumours (HT29) carried in nude mice. As shown in Table 2, a dose-dependent decrease in AAT activity was obtained, and a 75-80% reduction was seen in mice given four intraperitoneal injections of 110 mg/kg body weight. This reduction should be sufficient to examine the importance of the AAT protein in the sensitivity of mammalian tissues and tumours to the toxic, mutagenic and carcinogenic effects of alkylating agents.

### Repair of *O*<sup>6</sup>-methylguanine in oligodeoxynucleotides

The ability of mammalian and *E. coli* AATs to repair *O*<sup>6</sup>-methylguanine in relatively short oligodeoxynucleotides was tested using 4-mers, 6-mers and 12-mers. Initial experiments were carried out with high concentrations (50 units) of the *E. coli* AAT and 25 pmol of

this was the case was obtained by incubation of the AAT protein with 0.4 mM *O*<sup>6</sup>-methyl[8-<sup>3</sup>H]-guanine for 2-3 h. This resulted in loss of AAT activity with concomitant production of [8-<sup>3</sup>H]guanine (Dolan *et al.*, 1985a). The demethylation of *O*<sup>6</sup>-methylguanine and consequent inactivation of the AAT protein by reaction with the free-base *O*<sup>6</sup>-methylguanine occurs at a much lower affinity and a rate many thousands of times slower than the reaction with *O*<sup>6</sup>-methylguanine in DNA (Dolan *et al.*, 1985a; Yarosh *et al.*, 1986). Therefore, *O*<sup>6</sup>-methylguanine is not active as a competitive inhibitor of the AAT reaction with alkylated DNA, but pretreatment with it can be used to deplete cells of their AAT content.

The decrease in AAT activity brought about by exposure of cell cultures to *O*<sup>6</sup>-methylguanine is accompanied by an increased sensi-

**Table 2. Effect of *O*<sup>6</sup>-methylguanine treatment on AAT *in vivo***

Number of doses of <i>O</i> <sup>6</sup> -methylguanine	Tissue examined (fmol/mg)	AAT activity	% Control
0	Liver	57 ± 17	100
1	Liver	42 ± 5	74
2	Liver	25 ± 11	43
3	Liver	22 ± 13	39
4	Liver	14 ± 5	24
0	HT 29 tumour	134 ± 18	100
4	HT 29 tumour	27 ± 2	20

*O*<sup>6</sup>-Methylguanine was dissolved in 0.9% sodium chloride and given to female CD-1 mice or to female nude mice carrying the human colon HT29 tumour by intraperitoneal injection of 110 mg/kg body weight at hourly intervals. The animals were sacrificed 1 h after the last injection. Controls received 0.9% saline alone. Assays were carried out as described by Pegg *et al.* (1983).

*et al.*, 1983) was repaired slightly more slowly, 50% removal requiring 7 min at 0°C and 1.5 min at 37°C. The tetradeoxynucleotide 5'-dTm<sup>6</sup>GCA-3' (Fowler *et al.*, 1982) was also a substrate, but 45 min were required for 50% repair at 37°C (Scicchitano *et al.*, 1986).

A more sensitive assay method, which could also be used for mammalian AAT, was developed in which the oligodeoxynucleotides containing *O*<sup>6</sup>-methylguanine were labelled with <sup>32</sup>P by reaction with polynucleotide kinase and <sup>32</sup>P-ATP. After reaction with the alkyltransferase, the substrate and the resulting demethylated product were separated by reversed-phase HPLC. It was found that the reaction catalysed by AAT with both the 4-mers and the 12-mers was second order and that at 37°C the second-order rate constant for human HT29 tumour cell AAT was approximately 1.6 × 10<sup>9</sup> M/h for 5'-[<sup>32</sup>P]-dCGCm<sup>6</sup>GAGCTCGCG-3' (synthesized as described by Graves *et al.*, this volume) and 2.8 × 10<sup>7</sup> M/h for 5'-[<sup>32</sup>P]dTm<sup>6</sup>GCA-3'. The corresponding rate constants for *E. coli* AAT and these substrates were 2.5 × 10<sup>9</sup> M/h and 8.9 × 10<sup>7</sup> M/h, respectively.

These results and similar studies reported by Graves *et al.* (this volume) indicate that AAT can act on short oligodeoxynucleotides containing *O*<sup>6</sup>-methylguanine at a rate sufficient to permit convenient and highly sensitive assay methods. The oligodeoxynucleotides can be labelled with <sup>32</sup>P at very high specific activity (up to 5000 Ci/mmol). This very high specific activity more than compensates for the relatively slow rate of reaction obtained when the substrates and AAT are used at very low concentrations. It should be noted that it is not possible to run the reaction to completion under these conditions, and the amount of AAT protein must be calculated for the observed rate of reaction and the second-order rate constant. Assuming that 100 cpm is the minimum needed for accurate quantification, the sensitivity of the conventional assay procedure with <sup>3</sup>H-methylated DNA (which can be obtained with a specific activity of up to 3.5 Ci/mmol) has a detection limit of about 50 fmol, the limit with 4-mers is about 2 fmol and the limit with 12-mers is 0.1 fmol. The latter assay is therefore about 500 times more sensitive and should be suitable for use on very small samples, such as biopsy material and the limited amounts of purified cell types that can be obtained from some complex tissues. Investigations on AAT (including its cellular

the substrate in a total volume of 0.3 ml. After incubation, the content of *O*<sup>6</sup>-methylguanine was then determined using fluorescence detection after separation by high-performance liquid chromatography (HPLC) (Scicchitano *et al.*, 1986). It was found that dodecadeoxynucleotides of the sequence 5'-dCGNGAATTCm<sup>6</sup>GCG-3' (synthesized as described by Gaffney *et al.*, 1984) were all repaired very rapidly by the protein, with 50% repair in less than 15 sec at 0°C. The hexadeoxynucleotide 5'-dCGCm<sup>6</sup>GCG-3' (Kuzmich

distribution, regulation of its synthesis and variation in content from individual to individual in the human population), which are important in understanding the hazards posed by exposure to alkylating agents including *N*-nitroso compounds, have been hampered by the relatively low sensitivity of the available assays of its activity. It is well documented that human AAT levels can differ greatly between cell types, and there are known to be striking individual differences in the AAT content of tumorous and nonmalignant cells. The use of this ultrasensitive assay will enable estimates of this activity to be made accurately even when only very small samples are available.

#### **Acknowledgements**

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# DIET- AND AGE-DEPENDENT MODULATION OF O<sup>6</sup>-METHYLGUANINE-DNA METHYLTRANSFERASE LEVELS IN MOUSE TISSUES

M. Klaude & A. von der Decken

*The Wenner-Gren Institute for Experimental Biology, University of Stockholm,  
S-106 91 Stockholm, Sweden*

**Methylation of guanine in the O<sup>6</sup> position by *N*-nitrosodimethylamine is higher in liver DNA of mice fed a diet restricted in essential amino acids than in controls. A diminished content of the repair enzyme O<sup>6</sup>-methylguanine-DNA methyltransferase (AAT) may be responsible for the elevated level of O<sup>6</sup>-methylguanine observed after amino acid deficiency. A delayed repair of O<sup>6</sup>-methylguanine can be ascribed to diminished AAT activity in amino-acid-restricted mice. In adults, but not in subadults, the diminished AAT activity in liver correlated well with a 40% decrease in overall protein synthesis.**

In view of the link between alkylation of DNA-guanine, mismatching of the guanine residue in dsDNA and carcinogenesis, any effect of nutrition on the repair capacity of the cells seems of utmost importance. The enzyme responsible for the repair of O<sup>6</sup>-alkylguanine, AAT, should be present in cells in sufficient amounts to remove the methyl groups from the O<sup>6</sup>-position of guanine. Evidence has been provided that the repair of DNA is not the same in animals of different ages (Anisimov, 1985; Likhachev, 1985). Food utilization also differs with age, and responses to deficient diets may vary. To elucidate some of these points, the following studies have been carried out: (1) the effect of a balanced mixture of dietary amino acids on the content of AAT was determined in subadult and adult mice; and (2) the effect of a balanced mixture of dietary amino acids on overall protein synthesis *in vitro* was measured in the two groups of mice.

## **AAT activity and overall protein synthesis *in vitro***

Nutritional changes included a decrease in dietary methionine and cysteine to 10% the level of that in a balanced diet (Klaude & von der Decken, 1985). Tissue homogenates were ultrasonicated and centrifuged at 100 000 × *g* for 1 h. To determine AAT activity, the supernatant fraction was incubated with added DNA previously methylated with <sup>3</sup>H-*N*-methyl-*N*-nitrosourea (Margison, 1985). The transfer of methyl groups from the O<sup>6</sup>-position of guanine to the enzyme was assayed. The activity in liver per mg of tissue DNA of the control adults was 1.72-fold higher than that of the subadults (Table 1). With methionine-cysteine deficiency, the activity per mg of DNA in subadult liver decreased by 34% and that in the adult liver by 58%, resulting in similar activities for the two age groups. Taking into account the wet weight of liver, the total activity of AAT also decreased in both groups with amino acid deficiency (Table 1). No such marked age- or diet-related difference was observed in kidney, testis, brain or lung. The activity per mg of DNA was lower in these tissues than in liver. Testis, brain and lung contained less than half the activity of enzyme present in kidney.

Overall protein synthesis was determined by the incorporation of <sup>14</sup>C-leucine into protein, using the soluble factors from control liver and the ribosomes from control and amino acid-deficient groups. In liver of subadult mice, the specific activity per mg of DNA was unaffected by the dietary conditions (Table 2). In adult mouse liver, the experimental diet produced a significant decrease in activity per mg of DNA (Klaude & von der Decken, 1986).



**Table 1. Effect of diet and age on AAT activity, DNA content and wet weight of mouse liver<sup>a</sup>**

Age of animals	Diet	AAT content			
		fmol/mg protein	fmol/mg DNA	mg DNA/g wet wt	Liver wet wt
Subadult	Control	48.7 ± 1.85	1960.6 ± 64.0	2.20 ± 0.08	1.40 ± 0.09
	Deficient	46.8 ± 2.91	1295.3 ± 58.7 <sup>b</sup>	2.94 ± 0.19 <sup>c</sup>	0.85 ± 0.05 <sup>b</sup>
Adult	Control	50.4 ± 2.31	3371.2 ± 205.0	1.64 ± 0.11	1.61 ± 0.12
	Deficient	37.0 ± 1.23 <sup>b</sup>	1403.1 ± 39.3 <sup>b</sup>	2.51 ± 0.10 <sup>b</sup>	1.10 ± 0.05 <sup>c</sup>

<sup>a</sup>The results are mean values ± SE of 7-10 animals per dietary group; five determinations were made for each animal in the AAT assay, data were computed with the use of Student's *t* test.

<sup>b</sup>*p* < 0.001

<sup>c</sup>*p* < 0.01

### Conclusions

A high level of protein synthesis must be required to support the synthesis of repair enzymes, including AAT. The activity of AAT in cellular DNA appeared to be highest in liver as compared with kidney, testis, lung and brain (Craddock, 1984; Woodhead *et al.*, 1985). After feeding a balanced mixture of amino acids, overall protein synthesis in liver per mg of DNA was similar for subadult and adult mice, but the AAT activity in liver of the adults was higher. It may be argued that adult animals have been exposed to DNA damage

during a prolonged period of lifetime and require an increased repair capacity. With methionine-cysteine deficiency, the adults had decreased protein synthesis, but both the subadult and adult animals showed a diminished AAT activity, the decrease being more pronounced in the adults. There are four or five cysteine residues in a single AAT molecule (Demple *et al.*, 1982). A reduced amount of that amino acid will have a stronger effect on the synthesis of AAT than on proteins low in sulfur-containing amino acids.

Few data are available concerning the effect of dietary amino acids on DNA repair following damage induced by carcinogenic compounds (Klaude & von der Decken, 1985). The same

**Table 2. Effect of diet on protein synthesis by isolated ribosomes from liver of rats of two age groups<sup>a</sup>**

Diet	Leucine incorporated into protein (pmol/mg DNA)	
	Subadult	Adult
Control	1173.9 ± 61.2	1241.6 ± 80.2
Deficient	1244.6 ± 41.1	748.2 <sup>b</sup> ± 51.2

<sup>a</sup>The results are the mean values ± SE of four experiments; each experiment includes six determinations.

<sup>b</sup>*p* < 0.001

dietary conditions differ in their effects on body metabolism with the age of animals. Subadults take in food for maintenance metabolism and growth, while adults require food mainly for maintenance metabolism. The differences seen in the activity of AAT were associated with age when food conditions were optimal; the differences were abolished during amino acid deficiency. In both age groups, AAT capacity decreased, leaving the animals at an increased degree of probability of DNA-guanine mismatching in the dsDNA molecule.

### Acknowledgements

This work was supported by a grant from The Swedish Cancer Society (Project No. 1820-B86-04XB). The skilled technical assistance of Mrs Christina Olgar is gratefully acknowledged. The authors wish to thank Dr G.P. Margison for the gift of <sup>3</sup>H-DNA.

# O-ALKYL DEOXYTHYMIDINES ARE RECOGNIZED BY DNA POLYMERASE I AS DEOXYTHYMIDINE OR DEOXYCYTIDINE

B. Singer<sup>1,3</sup>, S.J. Spengler<sup>1</sup>, F. Chavez<sup>1</sup>, J. Sagi<sup>1,4</sup>, J.T. Kusmierek<sup>1,5</sup>,  
B.D. Preston<sup>2</sup> & L.A. Loeb<sup>2</sup>

<sup>1</sup>Donner Laboratory, University of California, Berkeley, CA 94720, USA;  
and <sup>2</sup>Department of Pathology, University of Washington Medical School,  
Seattle, WA 98195, USA

The *O*<sup>2</sup>- and *O*<sup>4</sup>-methyldeoxythymidine triphosphates (*O*-alkyl dTTP) can be used to substitute for dTTP in *Escherichia coli* DNA polymerase I (Pol I)-catalysed synthesis of poly[deoxyadenosine-deoxythymidine] (dA-dT). When incorporated into the polynucleotide, no detectable perturbation of structure occurred with even 20% *O*-methyldeoxythymidine in place of dT. However, on replication of such polymers with Pol I, significant amounts of deoxyguanosine triphosphate (dGTP) were incorporated, as well as high levels of deoxyadenosine triphosphate (dATP), indicating tautomer-like behaviour. Higher homologues, such as *O*<sup>4</sup>-ethyl (e<sup>4</sup>) dTTP or *O*<sup>4</sup>-isopropyl (ip<sup>4</sup>) dTTP, could also replace dTTP, but with lower efficiency. Nevertheless, their presence, like *O*<sup>4</sup>-methyl (m<sup>4</sup>) dT substitutions, caused transitions as well as inhibiting enzyme digestion with a variety of 3' nucleases, particularly to the 3' → 5' exonuclease activity (proofreading) of polymerases. Further proof of mutagenicity comes from site-directed experiments placing m<sup>4</sup>dT or e<sup>4</sup>dT in place of dT at position 587 in am3 of φX174, in which all revertants sequenced had A → G transitions. This implies that, since m<sup>4</sup>dT and e<sup>4</sup>dT are poorly repaired in eukaryotes, it is likely that they will remain in the DNA and lead to effects on enzyme activity, as well as mutations which contribute to the carcinogenicity of *N*-nitroso compounds.

The *O*<sup>2</sup>- and *O*<sup>4</sup>-alkyl pyrimidines are formed by carcinogenic *N*-nitroso compounds and, *in vivo*, are poorly repaired, particularly in cells which become tumorigenic (Singer *et al.*, 1981; Swenberg *et al.*, 1984; Richardson *et al.*, 1985). Since one aspect of their biological activity is the ability to mispair, or to be misincorporated, during replication, we have studied how *O*<sup>2</sup>-methyl (m<sup>2</sup>), m<sup>4</sup>-, e<sup>4</sup>- and ip<sup>4</sup>dT affect DNA structure, fidelity and enzyme recognition.

## Polymerase fidelity with *O*-alkyl pyrimidines

Polymerases deal with modified nucleosides in multiple ways. We consider here the recognition and utilization of the precursor triphosphates in DNA replication and recognition of the incorporated nucleoside during replication *in vitro* and *in vivo*.

<sup>3</sup>To whom correspondence should be addressed

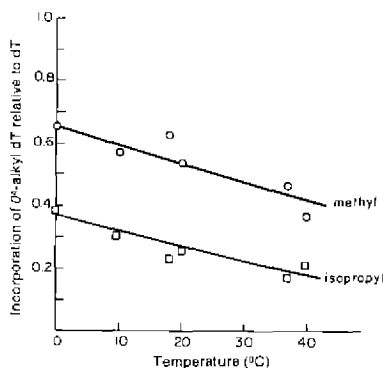
<sup>4</sup>Present address: Central Research Institute for Chemistry, Hungarian Academy of Sciences, H-1525 Budapest, Hungary

<sup>5</sup>Present address: Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw 02532, Poland

We have found that when dTTP is replaced totally or in part by the *O*-alkyl analogues, Pol I can utilize these derivatives, but at a slower rate (Singer, B. *et al.*, 1983, 1986a,b). The  $K_m$ 's of utilization, using poly(dA-dT) as template/primer, were found to be: dTTP, 0.7  $\mu$ M; m<sup>4</sup>dTTP, 5  $\mu$ M; e<sup>4</sup>dTTP, 11  $\mu$ M; ip<sup>4</sup>dTTP, 33  $\mu$ M (Singer, B. *et al.*, 1986b).

In addition, synthesis in the absence of dTTP terminates at significantly lower levels than normal. Studies of the relative rate of incorporation of these modified dTTPs, compared to dTTP, support our hypothesis that the alkyl derivative forms a secondary structure with dA and leads to the production of a poor primer terminus which apparently does not allow further replication. At temperatures lower than those used under standard conditions for replication with Pol I, the binding stability of the terminus containing *O*-alkyl dT is increased, leading to relatively higher synthesis (Singer, B. *et al.*, 1986b; Fig. 1).

**Fig. 1. Relative incorporation of nucleotides into DNA by DNA polymerase I in the presence of m<sup>4</sup>dTTP and *O*<sup>4</sup>-ipdTTP, compared to that with dTTP, as a function of incubation temperature**



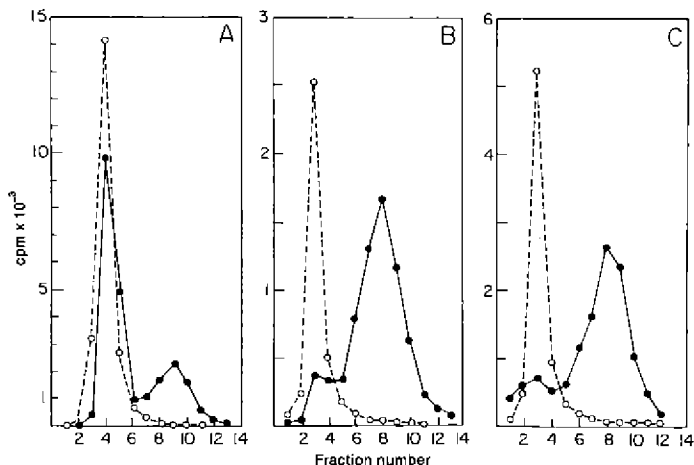
The level of dTTP incorporation is defined as 1.0 for each temperature. Deoxycytidine triphosphate, deoxyguanosine triphosphate and deoxyadenosine triphosphate were also present.

In a later section we discuss the difficulty in hydrolysing polymers containing *O*<sup>4</sup>-alkyl dT; this could lead to erroneously high estimates of misincorporation. *O*<sup>2</sup> modification of dT leads to fewer transitions during replication than *O*<sup>4</sup> alkylation. This tautomer-like behaviour, particularly of m<sup>4</sup>dT, is similar to that observed for a true tautomer, *N*<sup>4</sup>-hydroxycytidine, which exists in equilibrium as the keto and enol forms. The ability of m<sup>4</sup>dT to substitute for dC depends on the replication system: it has been observed *in vivo* using  $\phi$ X174 (Preston *et al.*, 1986a,b), as well as in translation and codon-anticodon binding studies (Singer, 1982).

Another aspect of preserving fidelity is the proofreading function of prokaryotic polymerases. We tested whether or not the primer terminus containing m<sup>4</sup>T:A pairs was rapidly excised by the 3'→5' exonuclease of the Klenow fragment of Pol I or T4 polymerase. We found that the presence of m<sup>4</sup>dT, at or near the 3' end, prevented the normal degradation to mononucleotides which occurs in the absence of deoxynucleoside triphosphates (Singer, 1986; Fig. 2).

Once the modified nucleoside is incorporated into DNA, or present as a result of direct modification, the alternative aspects of replication can be examined. Under these circumstances, Pol I recognized the *O*<sup>2</sup>- or *O*<sup>4</sup>-alkyl dTs primarily as dT but also, to varying extents as dC, thus causing significant numbers of transitions (e.g., 1 dG/10-100 m<sup>4</sup>dT) (Singer, B. *et al.*, 1983, 1984, 1986a). Estimates of dG misincorporation have been reported by Saffhill (1985) to be as high as 0.8 dG/m<sup>4</sup>dT. However, accurate estimates of misincorporation depend critically on analyses of polymers containing modified bases.

Fig. 2. Products of DNA polymerase-associated exonuclease digestion of polydA-dT (●) and poly(dA-dT, O<sup>4</sup>-methyl dT)(○) containing <sup>3</sup>H-labelled dA after Biogel P-150 separation of polymer (peak at fraction 4) and 5'-nucleoside monophosphate (5'-NMP) in the later peak



Identification of 5'-NMP as the sole component in this peak was by thin-layer chromatography. (A) and (B), treated with increasing amounts of the Klenow fragment and (C) with T4 polymerase under the same conditions as (B)

### In-vivo mutagenesis studies

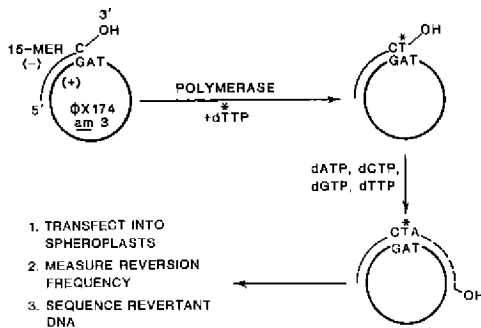
An emerging technique, site-directed mutagenesis, which has the potential to help elucidate the mechanism of both the misincorporation of modified dNTPs during replication *in vitro* and the mutation that occurs upon replication *in vivo*, has been utilized to study O-alkyl dTs. Figure 3 gives the essential elements of a protocol in which a primer pentadecamer (15-mer) terminating in *am3* of  $\phi$ X174 was extended *in vitro*, using Pol I, to form a 16-mer which could then be extended with all four dNTPs to the replicative form of the phage (Preston *et al.*, 1986a,b). When transfected into *E. coli* spheroplasts, this site-specifically modified DNA generated reversion mutants. Sequencing of revertant progeny DNA showed that every revertant had an A  $\rightarrow$  G transition at the position at which the O<sup>4</sup>-alkyl dTTP had been incorporated (Preston *et al.*, 1986b).

### Effect of O<sup>4</sup>-alkyl dT on polymer and DNA structures

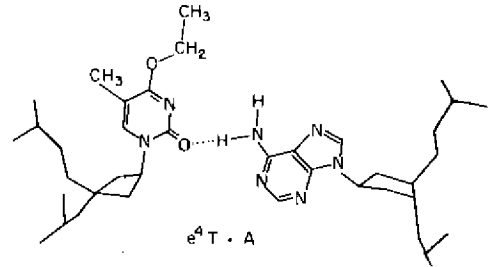
The incorporation into polynucleotides of nucleosides modified on non-base-pairing positions generally affects the secondary structure of polynucleotides. This is usually attributed to changes in stacking or the strength of base pairs. In contrast, the presence of m<sup>4</sup>dT opposite dA in poly(dA-dT) does not measurably change the  $T_m$ , the percent hypochromicity, or the width of the transition during melting. The same behaviour was seen for higher homologues in the O<sup>4</sup>-alkyl dT series, indicating that, although only a single hydrogen bond can be formed between O<sup>4</sup>-alkyl dT and dA, the alkyl group must be orientated so that it does not interfere with either the base pair or the planarity of the adjacent base pair. New information on the crystal structure of e<sup>4</sup>dT indicates that the ethyl

group lies *syn* to the N-3 and planar with the ring (Birnbaum *et al.*, 1986). The structure of the base pair shown in Figure 4 indicates that such an unusual pair can still be accommodated within the helical structure, with minimal distortion of the chain.

**Fig. 3. Protocol for site-specific insertion of dTTP analogues; T\* indicates the *O*-alkyl dTTP.**



**Fig. 4. Postulated e<sup>4</sup>T base pair with A (Singer, B. *et al.*, 1986b)**



The consistent transition data, AT → GC, is best explained by the ability of these derivatives to act as either dT or dC. An m<sup>4</sup>dT·dG pair which incorporates the crystallographic data has been proposed (Birnbaum *et al.*, 1986) which has an energy comparable to that of the e<sup>4</sup>dT·dA pair.

### Recognition of *O*<sup>4</sup>-alkyl dT by nucleases

One of the first effects we noted of the presence of *O*-alkyl dT was that poly(dA-dT) that contained e<sup>4</sup>- or ip<sup>4</sup>T could not readily be analysed for the content of *O*-alkyl dT by standard digestion procedures. Apparently, neither 3' nor 5' nucleases could digest these specific internucleotide bonds. This also appeared to be true for the φX174 primer extended by *O*-alkyl dT and the next correct nucleotide, dA. These data, together with data on the resistance to the 3' → 5' proofreading activity of polymerases (Fig. 2), may possibly indicate a significant biological effect. Not only recognition for repair, but also inhibition of the same enzymes, can occur when specific functional groups lie in either the major or minor groove. There is as yet no unequivocal evidence of a mammalian enzyme that recognizes the *O*-alkyl pyrimidine derivatives in the same manner as the prokaryotic repair systems (Hall & Karran, 1986).

### Acknowledgements

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**REPAIR OF SYNTHETIC OLIGONUCLEOTIDES  
CONTAINING O<sup>6</sup>-METHYLGUANINE, O<sup>6</sup>-ETHYLGUANINE  
AND O<sup>4</sup>-METHYLTHYMINE, BY  
O<sup>6</sup>-ALKYLGUANINE-DNA ALKYLTRANSFERASE**

**R.J. Graves, B.F.L. Li & P.F. Swann<sup>1</sup>**

*Courtauld Institute of Biochemistry, Middlesex Hospital Medical School,  
London W1P 7PN, UK*

**<sup>32</sup>P-Labelled self-complementary oligonucleotides containing O<sup>6</sup>-methylguanine, O<sup>6</sup>-ethylguanine, and O<sup>4</sup>-methylthymine have been synthesized and used as substrates for the DNA repair protein O<sup>6</sup>-alkylguanine-DNA alkyltransferase (AAT). The reaction was second-order with rate constants of  $2.6 \times 10^7 \text{ M}^{-1}\text{sec}^{-1}$  for O<sup>6</sup>-methylguanine,  $2.6 \times 10^4 \text{ M}^{-1}\text{sec}^{-1}$  for O<sup>6</sup>-ethylguanine and  $2.5 \times 10^3 \text{ M}^{-1}\text{sec}^{-1}$  for O<sup>4</sup>-methylthymine. These oligomers should allow sensitive and specific assay of the mammalian enzyme.**

Of the 15 products of alkylation of DNA by carcinogenic nitrosamines, two, O<sup>6</sup>-alkylguanine and O<sup>4</sup>-alkylthymine, are believed to be the most important. The effectiveness of these carcinogens is moderated by DNA repair enzymes, and the amount of these enzymes may play an important role in defining the sensitivity of different species and of individual organs (Pegg, 1984; Singer, 1984). Thus, there is a pressing need to develop methods for the assay of DNA repair enzymes, particularly for the enzymes of human tissue.

This has proved especially difficult for O<sup>4</sup>-alkylthymine. This base is repaired by AAT of *Escherichia coli*, but there is dispute as to whether the analogous mammalian enzyme is capable of this repair (Becker & Montesano, 1985; Dolan & Pegg, 1985; Yarosh *et al.*, 1985). The difficulty in resolving this question comes from the inadequacy of the methods used for assay.

In this paper we report that synthetic oligonucleotides containing alkylated bases are substrates for *E. coli* AAT; and could be used for assay of the transferase. Because the assay measures the formation of the nonalkylated parent oligonucleotide from the alkylated oligomer, it is specific for AAT; and because the assay uses <sup>32</sup>P-labelled oligomers, it is several orders of magnitude more sensitive than any previous assay.

Self-complementary dodecanucleotides dCGC*me*GAGCTCGCG, dCGC*et*GAGCTCGCG, dCGCAAGCT*me*TGCG, and their respective nonalkylated 'parent' sequences were synthesized by the phosphotriester approach (Li *et al.*, this volume). Purified *E. coli* AAT (fraction V; 5 units/ $\mu$ l), was a generous gift from Dr P. Karran and Dr T. Lindahl (Demple *et al.*, 1982). The oligodeoxynucleotides were labelled with <sup>32</sup>P on the 5'OH using [ $\gamma$ -<sup>32</sup>P]-ATP and T4 polynucleotide kinase, with minor adaptation of the published procedure (Maniatis *et al.*, 1982). Chromatographic analysis showed that this method produces complete phosphorylation of the oligomers.

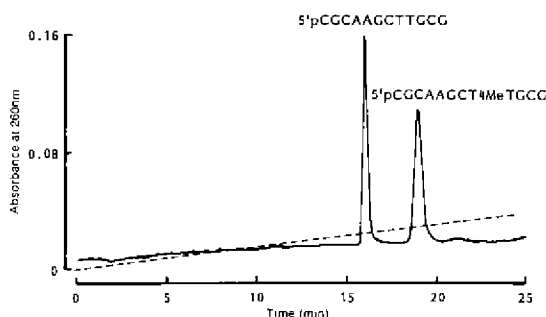
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<sup>1</sup>To whom correspondence should be addressed

Removal of the alkyl groups from dodecadeoxynucleotides containing *O*<sup>6</sup>-ethylguanine and *O*<sup>4</sup>-methylthymine was assayed using 10-15 pmol of *E. coli* AAT and 10-50 pmol of oligomer in 0.3 ml 50 mM Tris-HCl pH 7.6, 50  $\mu$ M spermidine, 10 mM dithiothreitol, 1 mM EDTA and 50  $\mu$ g/ml bovine serum albumin, and 0.1-0.5 pmol protein and 0.5-1.5 pmol oligomer in 3-12 ml buffer for *O*<sup>6</sup>-methylguanine. Reaction kinetics were followed by quenching the reaction with 0.1  $A_{260nm}$  units of the respective phosphorylated but nonradioactive alkylated oligodeoxynucleotide. The same amount of the phosphorylated nonradioactive parent oligodeoxynucleotide was also added to act as a carrier and optical marker for the high-performance liquid chromatography analysis. These quenched samples were injected without further treatment onto a Radial-PAK C18 cartridge (Waters, Inc.) at 45°C, and eluted at 3 ml/min with 0.35 M  $KH_2PO_4$  in water pH 6.3 with an increasing gradient (1%/min) of buffer B (0.35 M  $KH_2PO_4$  pH 6.3 in 33% acetonitrile).

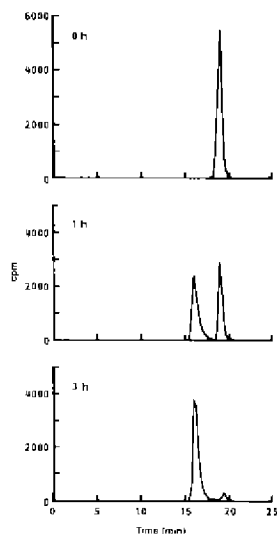
All the sequences containing alkylated bases could be separated from their nonalkylated parent sequences by reverse-phase high-performance liquid chromatography in phosphate buffer. Figure 1 shows the chromatographic separation of the *O*<sup>4</sup>-methylthymine dodecamer from its parent. Incubation of the alkylated parent with excess *E. coli* AAT removed all the alkyl groups, and the alkylated sequence was converted entirely back to the parent. Figure 2 shows the removal of methyl groups from <sup>32</sup>P-labelled *O*<sup>4</sup>-methylthymine dodecamer.

**Fig. 1. Separation of parent dodecamer from *O*<sup>4</sup>-methylthymine dodecamer by reverse-phase high-performance liquid chromatography**

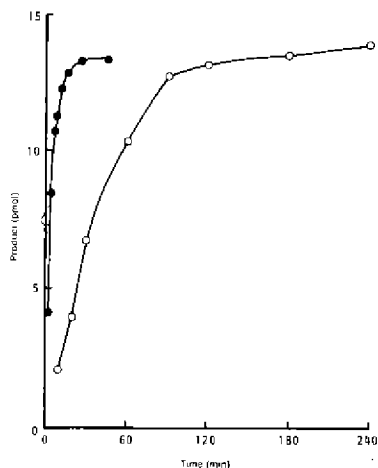


The rate of removal of the alkyl groups from the *O*<sup>6</sup>-ethylguanine dodecamer and the *O*<sup>4</sup>-methylthymine dodecamer for nanomolar concentrations of enzyme and oligomer are shown in Figure 3. The methyl groups were removed extremely rapidly from the *O*<sup>6</sup>-methylguanine dodecamer, but the kinetics could be followed by assaying at picomolar concentrations of enzyme and oligomer.

**Fig. 2. Formation of parent dodecamer from <sup>32</sup>P-labelled *O*<sup>4</sup>-methylthymine dodecamer by incubation with excess *E. coli* AAT**



**Fig. 3. Kinetics of removal by AAT of ethyl groups from *O*<sup>6</sup>-ethylguanine-containing oligomer (●) and *O*<sup>4</sup>-methylthymine-containing oligomer (○)**



Each reaction contained 14 pmol AAT and 45 pmol oligomer.

The initial rates of removal showed second-order kinetics, with the rate constant for removal of methyl from the *O*<sup>6</sup>-methylguanine dodecamer being  $2.6 \times 10^7 \text{ M}^{-1}\text{sec}^{-1}$ , that for removal of ethyl from the *O*<sup>6</sup>-ethylguanine dodecamer being  $2.6 \times 10^4 \text{ M}^{-1}\text{sec}^{-1}$ , and that for removal of methyl from *O*<sup>4</sup>-methylthymine dodecamer being  $2.5 \times 10^3 \text{ M}^{-1}\text{sec}^{-1}$ . These differences in the relative rate of removal of the alkyl group from the different alkylated bases in oligonucleotides mimic the differences in removal of these bases from alkylated DNA.

The level of DNA repair enzymes is important in conferring resistance to carcinogens and to chemotherapeutic agents such as chloroethylnitrosoureas (Brent *et al.*, 1985). Thus, there is a need for reliable assays of DNA repair enzymes in human tissues, both from the point of view of our understanding of the role of nitrosamines in human cancer and for strategies of chemotherapy. These oligonucleotides may prove to be the basis of these assays. Assay using alkylated DNA or polynucleotides has the disadvantage that many other alkylated products are invariably present. This is particularly true for *O*<sup>4</sup>-methylthymine, which represents only 0.06% of the methylation products of DNA by nitroso compounds. This proportion can be increased by alkylating poly (dT) or poly (dAdT), but neither of these polymers forms normal B-form DNA helices. In addition, the sensitivity of assays based on alkylated DNA is limited by the specific activity of the <sup>3</sup>H alkylating agents used in their preparation. Currently, this is about 3Ci/mmol. By contrast, [ $\gamma$ -<sup>32</sup>P]-ATP at 5000 Ci/mmol is readily available to anyone working in an institute with a molecular biology department.



**SYNTHESIS AND STRUCTURAL STUDIES BY  
NUCLEAR MAGNETIC RESONANCE OF  
DODECADEOXYNUCLEOTIDES CONTAINING  
*O*<sup>6</sup>-METHYLGUANINE, *O*<sup>6</sup>-ETHYLGUANINE  
AND *O*<sup>4</sup>-METHYLTHYMINE**

**B.F.L. Li<sup>1</sup>, P.F. Swann<sup>1</sup>, M. Kalnik<sup>2</sup> & D.J. Patel<sup>2</sup>**

*<sup>1</sup>Courtauld Institute of Biochemistry, Middlesex Hospital Medical School,  
London W1P 7PN, UK; and <sup>2</sup>Department of Biochemistry and Biophysics,  
College of Physicians and Surgeons, Columbia University, New York,  
NY 10032, USA*

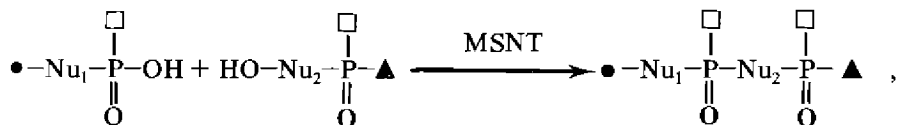
Self-complementary dodecadeoxynucleotides containing either one *O*<sup>6</sup>-methylguanine (*O*<sup>6</sup>-meGua), one *O*<sup>6</sup>-ethylguanine (*O*<sup>6</sup>-etGua) or one *O*<sup>4</sup>-methylthymine (*O*<sup>4</sup>-meT) have been synthesized. They form double-stranded DNA in solution. The structures of these DNA helices containing *O*<sup>6</sup>-meGua:cytosine (C), *O*<sup>6</sup>-meGua:thymine (T), *O*<sup>6</sup>-etGua:C and Gua:*O*<sup>4</sup>-meT base pairs have been investigated by nuclear magnetic resonance (NMR). All these modified bases stack into the helix with the normal anti-glycosidic torsion angle; only in the helix containing an *O*<sup>6</sup>-etGua:C base pair was there evidence of significant distortion of the DNA structure. NMR did not show a strong hydrogen bond between N1 of Gua and N3 of *O*<sup>4</sup>-meT in the Gua:*O*<sup>4</sup>-meT base pair, or between N1 of *O*<sup>6</sup>-meGua and N3 of T in the *O*<sup>6</sup>-meGua:T base pair. This casts doubt on the previously accepted structures for *O*<sup>4</sup>-meT:Gua and *O*<sup>6</sup>-meGua:T mispairs.

The most important DNA modification in nitrosamine carcinogenesis is the alkylation of *O*<sup>6</sup> of guanine (Gua) residues and *O*<sup>4</sup> of thymine (T) residues. To gain insight into the base-pairing properties of these alkylated bases and the conformational changes in DNA resulting from their presence, and also into the mechanism of action of DNA repair enzymes, dodecadeoxynucleotides containing *O*<sup>6</sup>-meGua, *O*<sup>6</sup>-etGua and *O*<sup>4</sup>-meT have been synthesized. The sequences were self-complementary and formed double-stranded DNA in solution. Oligodeoxynucleotides containing *O*<sup>6</sup>-meGua have been prepared before (Fowler *et al.*, 1982; Gaffney *et al.*, 1984), but we believe that this is the first report of the synthesis of oligodeoxynucleotides containing *O*<sup>6</sup>-etGua and *O*<sup>4</sup>-meT.

The *O*<sup>4</sup>-meT required for oligonucleotide synthesis was prepared by treating the 4-(3-nitro-1,2,4-triazolo) derivative of 3',5'-di-*O*-methoxyacetylthymidine with 1,8-diazabicyclo-[5.4.0]undec-7-ene in methanol solution. 2-*N*-Phenylacetyl-6-*O*-alkyl-2'-deoxyguanosine for oligonucleotide synthesis was prepared by treating the 6-*O*-(mesitylene-2-sulfonyl) derivative of 3',5'-diacetyl-2-*N*-phenylacetyl-2'-deoxyguanosine with *N*-methylpyrrolidine and then displacing the methylpyrrolidine with alkoxide ions (ROH/DBU). Very pure products were obtained in high yield. As is normally done, the amino groups of 2'-deoxycytidine and 2'-deoxyadenosine were protected with benzoyl groups. It is not usual to protect the *O*<sup>6</sup> of deoxyguanosine or the *O*<sup>4</sup> of thymidine, but, since side reactions with these groups have been reported (Reese & Skone, 1984), 2'-deoxyguanosine was protected as the

2-*N*-acetyl-*O*<sup>6</sup>-(3-chlorophenyl) derivative and thymidine as the *O*<sup>4</sup>-phenyl. To avoid attack on the alkyl group during deprotection, the phenylacetyl group was used for *N*(2) protection of the alkylguanine; this can be rapidly removed with ammonia irrespective of the alkyl group on guanine.

Oligonucleotides were synthesized from protected 3'-nucleotides by the phosphotriester approach in solution. Protected 3'-nucleotides were condensed to form dimers, which were then condensed to form the three tetramers from which the dodecanucleotides were made. The typical reaction for the formation of the dimers can be represented schematically:



where  $\bullet$  represents a 5'-*O*-acetyl or 5'-*O*-(9-phenylxanthen-9-yl) 'pixyl' group;  $\square$ , a 2-chlorophenyl group protecting phosphate;  $\blacktriangle$ , the 2-cyanoethyl group used for transient phosphate protection; and MSNT is 1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazole. The 5' protecting group or the 2-cyanoethyl protecting the 3' phosphorus was then removed and the tetramers formed by condensing (as in the scheme) one dimer with a free 5'-OH and another with a free 3'-phosphodiester.

The synthesis of oligomers containing *O*<sup>4</sup>-meT presented particular problems. The pixyl protecting the 5'-OH is normally removed with a protic acid, but, as *O*<sup>4</sup>-meT is susceptible to hydrolysis, zinc bromide had to be used for 5' deprotection of intermediates containing *O*<sup>4</sup>-meT residues. The instability of *O*<sup>4</sup>-meT also presented problems when the protecting groups were removed from the bases, internucleotide linkages, and the 5' and 3' termini at the end of the synthesis. Normally, ammonia is used to remove *N*-acyl protecting groups, and acid to remove the 5' protecting group, but *O*<sup>4</sup>-meT residues are converted to 5-methylcytosine by ammonia. However, these problems were overcome by use of methoxide ions (1,8-diazobicyclo[5.4.0]undec-7-ene/methanol) rather than ammonia, and by protecting the terminal 3' and 5' of the dodecamers with the base-sensitive acetyl group. The dodecamers were pure, as judged by high-performance liquid chromatography of both the oligomer and the nucleosides obtained by enzymic digestion. An interesting demonstration of their purity is that the alkylated oligomers can be completely converted to the parent oligomers by the DNA repair enzyme *O*<sup>6</sup>-alkylguanine-DNA alkyltransferase (see Graves *et al.*, this volume).

The structure of these oligomers is being investigated by nuclear magnetic resonance (NMR). The first oligonucleotide structures were solved by X-ray crystallography, which gives precise measurements, but most oligomers have failed to crystallize or have given crystals that do not diffract. NMR is a less developed technique, but it is evolving rapidly; measurements are made in solution and all sequences can be studied. Since it is not possible to give the results of the NMR studies on these modified oligomers in detail, we shall concentrate on three questions that illustrate its application:

- (1) Is the helix containing the modified bases right-handed with the nucleotides having the *anti* configuration as in B-DNA?
- (2) Is the widely accepted mispaired structure between *O*<sup>6</sup>-alkylguanine and T, and between *O*<sup>4</sup>-alkylthymine and Gua, correct?
- (3) What is the position of the purine and the ethyl groups when *O*<sup>6</sup>-etGua is incorporated into DNA?

If the DNA is a *right-handed* helix, the C8 proton of a purine or the C6 proton of a pyrimidine is less than 0.45 nm, from the H1' of its own sugar, and from the H1' of the nucleotide on the 5' side. If a proton is irradiated at its NMR resonance frequency, the NMR signal of protons within 0.45 nm will be affected. This is called nuclear Overhauser enhancement (NOE). The intensity of the NOE is related to the proximity of each proton to the irradiated proton. Therefore, if the helix is right handed, every purine-H8/ pyrimidine-H6 (except the 5' terminal base) will have an NOE to two H1'.

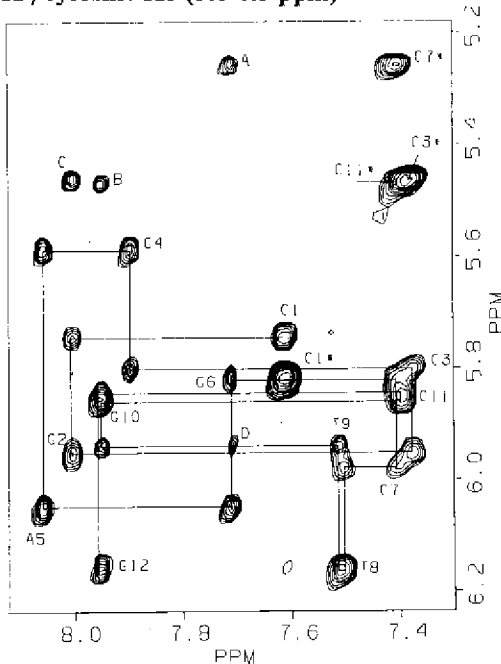
Figure 1 is a two-dimensional NOE (NOESY) spectrum of:

C1-G2-C3 ---- G4-A5-G6-C7-T8-meT9-G10-C11-G12

G12-C11-G10-meT9-T8-C7-G6-A5---G4-C3-G2-C1

covering the range of chemical shifts of purine H8 and pyrimidine H6 (7-8 ppm), and of H1' and cytosine H5 (4.5-6 ppm) in DNA. C1 marks the NOE between C1-H6 and C1-H1'. As C1 is the 5' terminus, there is no nucleotide on its 5' side, but C1-H1' has an NOE with G2-H8. G2-H8 has a NOE with G2-H1' (follow the line vertically to the peak marked G2). G2-H1' has an NOE to C3-H6, which has an NOE to C3-H1' (marked C3) etc. As you can see, the line can be followed through every nucleotide; therefore, the helix is right handed.

**Fig. 1. Two-dimensional nuclear Overhauser enhancement spectrum (NOESY) of DNA sequence containing two *O*<sup>6</sup>-etGua:C base pairs showing the NOEs between purine H8/-pyrimidine H6 (7.2-8 ppm) and deoxyribose H1'/cytosine H5 (5.0-6.5 ppm)**



The NOEs between the base and its own sugar H1' are marked C1, G2, C3, etc; those between H5 and H6 of each cytosine are marked C1\*, etc. The DNA sequence is given in the text.

This spectrum also shows that every nucleoside has the *anti* conformation. In the *anti* conformation, the distance between purine H8/ pyrimidine H6 and sugar H1' is about 0.37 nm. In the *syn* conformation, it is about 0.24 nm, which is similar to the distance between cytosine H5 and H6. Thus, in the *anti* configuration, the magnitude of the NOE between purine H8/ pyrimidine H6 and its own H1' is less than that of the NOE between H5 and H6 of cytosine; in the *syn* configuration, the NOEs would be similar. Figure 1 shows that the NOE between purine H8/ pyrimidine H6 and the H1' of the same nucleoside (peaks marked C1, G2, C3, etc) are in every case much smaller than the NOE between cytosine H5 and H6 (marked C1\*, C3\*, etc); therefore, every nucleoside has the *anti* configuration.

The second question concerns the base-pairing properties of the alkylated bases. To explain the mutagenic properties of *N*-nitroso compounds, it has been proposed that *O*<sup>6</sup>-alkylguanine residues form base pairs with T residues in DNA in which the imino proton of T forms a hydrogen bond with N(1) of the alkylguanine. Similarly, *O*<sup>4</sup>-alkylthymine is thought to form a base pair with Gua residues, in which the imino

proton of Gua forms a hydrogen bond with N(3) of *O*<sup>4</sup>-alkylthymine (Abbott & Saffhill, 1977). NMR measurements do not support these structures. Hydrogen-bonded imino protons in DNA have a shift between 12 and 14 ppm downfield, but, in the sequence C1-G2-C3-*meG*4-A5-G6-C7-T8-T9-G10-C11-G12, which forms a double helix with an *O*<sup>6</sup>-*meGua*:T base pair, the chemical shift of the imino proton in this pair was 9.54 ppm. Similarly, in the sequence C1-G2-C3-G4-A5-G6-C7-T8-*meT*9-G10-C11-G12, which forms a double helix with a *Gua*:*O*<sup>4</sup>-*meT* base pair, the chemical shift of the imino proton in this pair was 8.70 ppm. The imino protons of the *Gua*:C pairs in these two sequences had shifts from 12.3-13.08 ppm; the A:T pairs, 13.92 and 14.09 ppm downfield. Similar observations on the *O*<sup>6</sup>-*meGua*:T pair have been made before (Patel *et al.*, 1986a,b).

The upfield shift of the imino protons in the *O*<sup>6</sup>-*meGua*:T and *Gua*:*O*<sup>4</sup>-*meT* pairs suggests that these protons are not involved in hydrogen bonds, or at least that the bonds must be very weak. This interpretation is supported by the observation that the chemical shift of these protons does not change greatly as the temperature is raised. These results call into question the currently accepted structures for *O*<sup>6</sup>-*meGua*:T and *Gua*:*O*<sup>4</sup>-*meT* pairs.

The previous pairing scheme for *Gua*:*O*<sup>4</sup>-*meT* has also been questioned by Brennan *et al.* (1986), who found by X-ray crystallography that in the crystal the *O*<sup>4</sup>-CH<sub>3</sub> of *O*<sup>4</sup>-methylthymidine is *syn* to N(3). This would interfere with the formation of an H-bond between N(3) of *O*<sup>4</sup>-*meT* and N(1) of *Gua* because of the reduced accessibility of N(3) in *O*<sup>4</sup>-*meT* and because of steric interaction between the *O*<sup>4</sup>-CH<sub>3</sub> group and the *O*<sup>6</sup> of *Gua*. Although the NMR studies are not yet complete and the exact conformation of *O*<sup>4</sup>-*meT* in the oligomer is not known, we did not observe a strong NOE between the *O*<sup>4</sup>-CH<sub>3</sub> and 5-CH<sub>3</sub> of *O*<sup>4</sup>-*meT*, suggesting that the *O*<sup>4</sup>-CH<sub>3</sub> is *syn* to N(3) in the oligomer as it is in the nucleoside.

The third question concerning the ability of NOESY spectra to show that all these alkylated nucleosides stack into the helix with the alkyl groups in the major groove of the DNA can be illustrated by studies on the sequence with an *O*<sup>6</sup>-*etGua*:C base pair:

C1-G2---C3-*etG*4-A5-G6-C7-T8---C9-G10-C11-G12

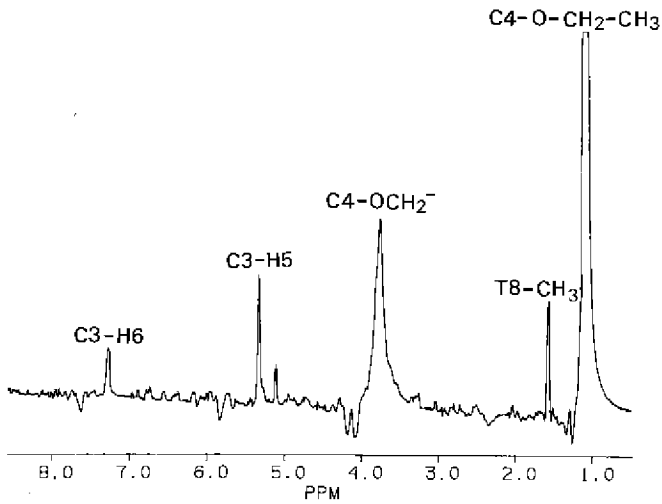
G12-C11-G10--C9-T8-C7-G6-A5-*etG*4-C3--G2--C1.

Figure 2 shows NOEs from the -CH<sub>3</sub> of the ethyl group to H5 and to H6 of the cytosine flanking it in the sequence (C3), and to the 5-CH<sub>3</sub> of the T (T8) in the opposite strand. C-H5 and T-CH<sub>3</sub> are in the major groove of DNA. The only clear NOEs from the -CH<sub>2</sub>- are to the -CH<sub>3</sub> of the ethyl and to C3-H5. These NOEs can be used to place the ethyl group in the DNA structure. Interestingly, this is the only sequence in which there was evidence that the presence of the alkylated base caused significant distortion of the DNA. This could be seen in the <sup>31</sup>P spectrum, in which there was a greater than normal dispersion of the resonance peaks. Eventually, NMR should be able to define these conformational changes because the estimates of distance given by NOE spectra can be used to elucidate the detailed structure of macromolecules by the technique of Distance Geometry or Molecular Dynamics.

#### Acknowledgements

We are grateful for the support of the Cancer Research Campaign and the Medical Research Council.

**Fig. 2. Nuclear Overhauser enhancement (NOE) spectrum from the  $-\text{CH}_3$  of  $O^6$ -etGua in the DNA sequence, showing that it is close to the methyl of the thymine in the opposite strand (T8) and to H5 and H6 of the adjacent cytosine (C3)**



The DNA sequence is given in the text.

## ORGAN SPECIFICITY, METABOLISM AND REACTION WITH DNA OF ALIPHATIC NITROSOMETHYLALKYLAMINES

P. Kleihues<sup>1</sup>, E. von Hofe<sup>1</sup>, I. Schmerold<sup>1</sup>, L.K. Keefer<sup>2</sup> & W. Lijinsky<sup>3</sup>

<sup>1</sup>Laboratory of Neuropathology, Department of Pathology, University of Zürich, 8091 Zürich, Switzerland; <sup>2</sup>Chemistry Section, Laboratory of Comparative Carcinogenesis, National Cancer Institute, Frederick Cancer Research Facility, Frederick, MD 21701, USA; and <sup>3</sup>LBI-Basic Research Program, Chemical Carcinogenesis Program, National Cancer Institute, Frederick Cancer Research Facility, Frederick, MD 21701, USA

Aliphatic nitrosomethylalkylamines are carcinogens with a remarkable organ specificity in rats, the principal targets being liver, oesophagus and bladder. We have determined the extent of DNA methylation in these tissues following a single oral dose (0.1 mmol/kg; 6-h survival) of each of 12 homologues, ranging from *N*-nitrosodimethylamine (NDMA, C1) to *N*-nitrosomethyldodecylamine (C12). Methylpurines (7- and *O*<sup>6</sup>-methylguanine; 7-meGua and *O*<sup>6</sup>-meGua) were determined by cation-exchange high-performance liquid chromatography with fluorescence detection. Highest levels of hepatic DNA methylation were found with NDMA (C1) and *N*-nitrosoethylmethylamine (NEMA, C2), the most potent hepatocarcinogens in this series. Concentrations of methylpurines in liver DNA decreased with increasing chain length from C1 to C5. Administration of the higher homologues (C6-C12) caused levels of DNA methylation which by themselves were considered too low to account for their hepatocarcinogenicity. In rat oesophagus, DNA methylation closely paralleled carcinogenicity, the most effective agents being the butyl and pentyl derivatives (C4 and C5). Levels of DNA methylation in bladder epithelium were close to the limit of detection (C6, C9, C10, C12) and there was no apparent correlation with carcinogenicity. It is concluded that initiation of malignant transformation by DNA methylation alone (through hydroxylation of the nitrosamine at the methylene  $\alpha$ -carbon) could be operative for C1-C5. For the higher homologues, this type of DNA modification is insufficient to explain the complex pattern of tissue specificity.

Carcinogenic nitroso compounds are characterized by their capacity to induce selectively a high incidence of malignant tumours in a wide spectrum of target tissues. The biological basis of organ-specific carcinogenesis is not yet fully understood, but several factors have been identified, including distribution of the parent carcinogen, tissue-specific bioactivation and DNA repair (Table 1). Investigations into these mechanisms have in the past been limited to a small group of carcinogens and a few selected target tissues (Langenbach *et al.*, 1983; Kleihues & Wiestler, 1986). This report summarizes data on the acute biochemical effects of asymmetric nitrosomethylalkylamines, which represent one of the most fascinating examples of a correlation between chemical structure and organ-specific carcinogenicity. DNA methylation was determined in target and nontarget tissues and correlated with tumorigenicity in chronic bioassay studies.

**Table 1. Factors involved in organ-specific tumour induction**


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— Type of DNA modification
— Extent of DNA modification
— Initial distribution of the parent carcinogen
— Bioactivation in target and nontarget tissues
— Interorgan shift of carcinogen metabolism
— Extrahepatic reactivation of conjugates
— DNA repair
— Cell division
— Tissue-specific tumour promotion
— Inherent tissue susceptibility (e.g., presence or accessibility of proto-oncogenes)

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**Carcinogenicity of nitrosomethylalkylamines in rats**

The pioneering work of Druckrey and co-workers (1967) has established that the most powerful oesophageal carcinogens are asymmetric nitrosamines with a methyl group as one of the alkyl moieties. In the homologous series of aliphatic nitrosomethylalkylamines, the only symmetrical agent, NDMA (C1), does not induce oesophageal neoplasms. The next asymmetric homologue, NEMA, still produces predominantly liver tumours but also a low incidence of oesophageal neoplasms. The higher homologues (*N*-nitroso-

propylmethyl- to *N*-nitrosohexylmethylamine) all produce predominantly or exclusively oesophageal tumours in rats. Lijinsky and coworkers (1981) have systematically investigated the organ specificity of long-chain nitrosomethylalkylamines (nitrosoheptylmethyl- to nitrosododecylmethylamine) and found that nitrosamines with odd-numbered carbon chains preferentially induced liver tumours, while even-numbered ones induced bladder cancer.

**DNA methylation by nitrosomethylalkylamines**

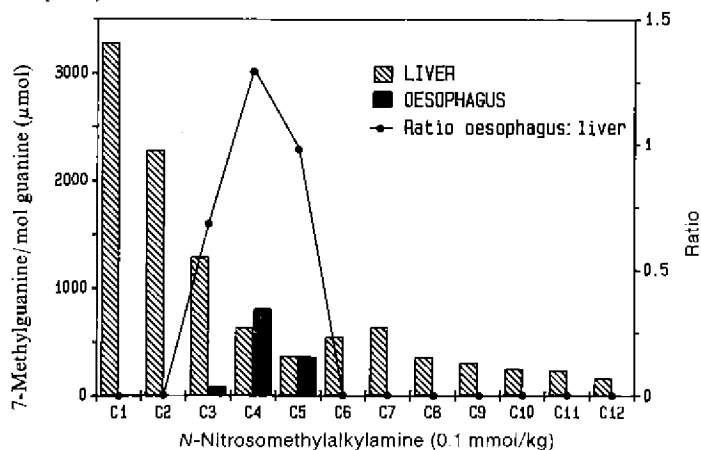
Unlabelled nitrosamines were administered in a single dose per gavage at 0.1 mmol/kg, and survival time was 6 h. Hepatic DNA was isolated by adsorption onto hydroxylapatite as described previously (von Hofe *et al.*, 1986a). Following partial hydrolysis in 0.1 N hydrochloric acid, purines were analysed by high-performance liquid chromatography on a strong cation exchange column (Partisil SCX) using a modification (von Hofe & Kleihues, 1986) of the procedure of Swenberg and Bedell (1982). Modified bases were detected by fluorescence at 370 nm with an excitation of 295 nm (Shimadzu RF-540 fluorophotometer).

The results obtained in liver and oesophagus are summarized in Figure 1. The extent of DNA methylation in rat liver is highest with NDMA (3300  $\mu\text{mol}$  7-meGua/mol guanine) and gradually decreases. The concentration of 7-meGua produced by an equimolar dose of *N*-nitrosopentylmethylamine (NPMA, C5) comprises 11% of that produced by NDMA. Long-chain nitrosomethylalkylamines (C6-C12) all produced a low extent of hepatic DNA alkylation (200-600  $\mu\text{mol}$  7-meGua/mol guanine). In contrast, oesophageal alkylation increased from values below the level of detection (C1, C2) to 800 and 365  $\mu\text{mol}$  7-meGua/mol guanine for *N*-nitrosobutylmethylamine (NBMA) and NPMA, respectively. In the case of longer-chain homologues (C6-C12), 7-meGua was not found. The detection limit for *O*<sup>6</sup>-meGua was somewhat lower owing to its strong fluorescence, and this modified base was detected with all compounds. The 7-meGua ratio for oesophagus/liver was highest for NBMA.

**DNA alkylation by *N*-nitrosoethylmethylamine**

The simplest asymmetric nitrosamine, NEMA, was studied to determine the ability of nitrosomethylalkylamines to carry out reactions with DNA other than methylation. In an

**Fig. 1. DNA methylation in liver and oesophagus of Fischer 344 rats 6 h following a single oral dose of a nitrosomethylalkylamine in the series from nitrosodimethylamine (C1) to nitrosododecylmethylamine (C12)**



NEMA was compared to that caused by an equimolar mixture of NDMA plus *N*-nitrosodiethylamine (NDEA). The total molar dose of NDMA plus NDEA was equal to the total molar dose of NEMA. While levels of *O*<sup>6</sup>-meGua in hepatic DNA from animals treated with NEMA were comparable to those in animals treated with an equimolar dose of NDMA plus NDEA, *O*<sup>6</sup>-etGua concentrations were roughly four times lower in rats treated with NEMA than in animals treated with NDMA plus NDEA (Fig. 2).

We next investigated the possibility that some pathway besides  $\alpha$ -C hydroxylation could be occurring which would yield DNA adducts other than methylation and ethylation products. The most likely alternative was  $\beta$ -C hydroxylation. That metabolism at this carbon atom may play a role in the carcinogenicity of this compound had been suggested by the altered tissue-specific carcinogenicity of  $\beta$ -trideuterated NEMA (Lijinsky & Reuber, 1980). 7-(2-Hydroxyethyl)guanine was indeed detected in hepatic DNA from rats treated with a single intraperitoneal dose (Fig. 3), but comprised less than 2% of the amount of 7-etGua present (von Hofe *et al.*, 1986b).

### Discussion and conclusions

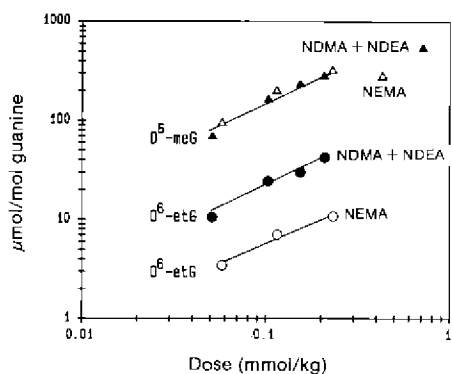
Interpretation of these experiments is limited by the fact that for most compounds, DNA analysis was restricted to the determination of the methyl purines, 7-meGua and *O*<sup>6</sup>-meGua. Comparison of data from experiments employing different protocols further complicates the interpretation, since large differences might accompany changes from gavage or intraperitoneal administration (bolus doses) to drinking-water administration (repeated, small doses), from males to females, or from one dose size to another. However, we feel that the results obtained allow the formulation of some tentative conclusions and hypotheses which may be further tested in a more comprehensive study of DNA modifications resulting from exposure to long-chain asymmetric nitrosamines.

initial study using NEMA <sup>14</sup>C labelled in either the ethyl or methyl group, both methylated and ethylated purines were found in hepatic DNA. The ratio of 7-meGua:7-ethylguanine (7-etGua) was approximately 180, indicating that this compound is much more effective in methylating hepatic DNA than it is in ethylating. If  $\alpha$ -C hydroxylation had occurred at a similar rate in the methyl and ethyl groups, a ratio of 68 would have been expected (von Hofe *et al.*, 1986a).

In a subsequent experiment (von Hofe & Kleihues, 1986), the amount of methylation and ethylation by

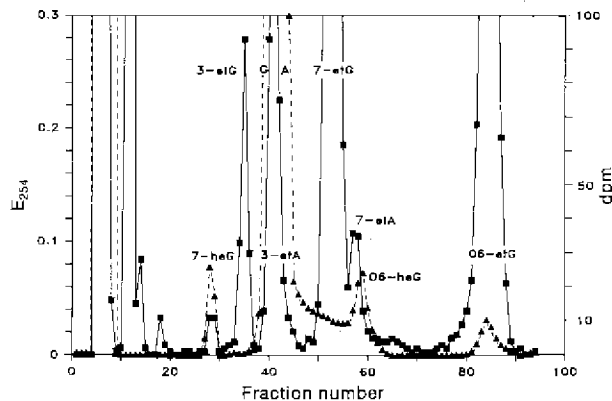


**Fig. 2. Dose-dependent formation of *O*<sup>6</sup>-alkylguanines after a single intraperitoneal dose of NEMA or NDMA plus NDEA**



From von Hofe & Kleihues (1986)

**Fig. 3. Ion-exchange chromatographic profile of hepatic DNA purines from a male Fischer 344 rat injected intraperitoneally with 6.3 mg/kg [1-ethyl-<sup>14</sup>C]NEMA; ▲, absorbance (254 nm); ■, radioactivity (dpm)**



From von Hofe *et al.* (1986b)

ation only with those nitrosomethylalkylamines that are exclusively or predominantly bioactivated by initial  $\alpha$ -C hydroxylation at the alkyl group opposite the methyl. The results shown in Figure 1 suggest that this occurs for C1 and C2 in rat liver, and for C4 and C5 in the oesophagus.

(2) *DNA methylation occurs in rat liver and oesophagus with the entire homologous series of nitrosomethylalkylamines investigated*, although in oesophageal DNA, levels of methylpurines were close to the limit of detection for C1 (NDMA), C2 (NEMA), and C6 to C12. This indicates that  $\alpha$ -C hydroxylation is operative in both tissues but its extent is strongly dependent on the configuration of the alkyl chain.

(1) *DNA methylation resulting from a single hydroxylation step is most likely if it occurs in the  $\alpha$ -C position of the opposite alkyl chain.* Methylation can also occur subsequent to enzymic attack at any other C-atom. However, this often leads to more polar intermediates, which are generally more stable than  $\alpha$ -C hydroxy intermediates. This increased stability may allow for redistribution, excretion and further enzymic or spontaneous modifications. We therefore conclude that the most effective pathway for the production of methyl adducts is initial alkyl  $\alpha$ -C hydroxylation, since the resulting intermediate is extremely short-lived and will rapidly react with available nucleophiles. This view is supported by our studies on NEMA. Although there was evidence of extensive hydroxylation at the  $\beta$ -group, this led to only a very small amount of DNA hydroxyethylation. Similarly, Mirvish *et al.* (1985) identified four hydroxy derivatives of NPMA (C5), but only those resulting from attack at positions C2 to C5. It was concluded that the  $\alpha$ -hydroxy intermediate, although probably responsible for DNA methylation, was too short-lived for chemical detection. Accordingly, one would expect a high extent of methyl-

(3) *DNA methylation in rat liver decreases with increasing length of the alkyl chain.* From the data in Table 1, this is obvious for C1 (NDMA) to C5 (NPMA). The low levels of 7-meGua produced by C5 to C12 could indicate that enzymic  $\alpha$ -C hydroxylation is less effective for these agents than for C1-C4. The alternative interpretation is that the liver does not contain P450 isozymes capable of  $\alpha$ -hydroxylating nitrosomethylalkylamines with a chain length greater than four. In this case, DNA methylation would first require a shortening of the alkyl chain.

(4) *Initiation of hepatocarcinogenesis by DNA methylation alone can be assumed only for C1 (NDMA) and C2 (NEMA).* These agents are strong hepatocarcinogens and produce 7-meGua and  $O^6$ -meGua levels of more than 2000  $\mu\text{mol}$  and 200  $\mu\text{mol/mol}$  guanine per 0.1 mmol nitrosamine/kg, respectively. *N*-Nitrosopropylmethylamine (C3) produces less than 100  $\mu\text{mol}$   $O^6$ -meGua/mol guanine at this dose and is not known to produce liver cancer in rats. All higher homologues (C4-C12) produce substantially less DNA methylation than does C3, although several of these (C6-C9, C11) induce a high incidence of hepatic carcinomas following chronic oral administration. This strongly suggests that the initiation of liver carcinogenesis by these agents requires DNA modifications other than, or in addition to, methylation.

(5) *In rat oesophagus, DNA methylation by nitrosomethylalkylamines closely parallels carcinogenicity.* From the data summarized in Figure 1, it is concluded that the oesophageal mucosa of rats contains a P450 isozyme capable of metabolizing asymmetric nitrosamines at the  $\alpha$ -methylene of alkyl groups bearing three to five C atoms. The propyl, butyl and pentyl derivatives appear to be most effectively metabolized in this way and all are potent oesophageal carcinogens. The other compounds studied produced neither oesophageal tumours nor extensive oesophageal DNA methylation, except for *N*-nitrosohexylmethylamine (C6) which is a fairly potent carcinogen in this organ although its capacity to methylate target organ DNA was low.

(6) *The amount of DNA methylation required to initiate liver carcinogenesis is considerably higher than that required to produce a similar incidence of oesophageal cancer.* Our studies suggest that any agent that produces 10 or more  $\mu\text{mol}$   $O^6$ -meGua/mol guanine per 0.1 mmol nitrosamine/kg is already a strong and often selective oesophageal carcinogen, i.e., at levels of DNA methylation 10-20 times lower than required to induce liver tumours. This hypothesis relies on our studies with NEMA, which showed that the levels of DNA adducts other than methylpurines were quantitatively very low (ethylpurines) or negligible (hydroxyethylpurines). Hepatic tumour induction by asymmetric nitrosomethylarylamines (e.g., *N*-nitrosomethylbenzylamine) is also low, although, in the view of Silinskas *et al.* (1984), the extent of DNA ethylation should be sufficient to induce liver tumours in addition to oesophageal carcinomas.

(7) *The present data on DNA methylation are insufficient to explain the selective induction of liver and bladder carcinomas by long-chain odd- and even-numbered nitrosomethylalkylamines, respectively.* We did detect small amounts (approximately 5  $\mu\text{mol/mol}$  guanine) of  $O^6$ -meGua in bladder DNA following administration of *N*-nitrosododecylmethylamine (C12) and trace amounts after exposure to C6, C9, C10 and C12 (data not shown). Since this does not correlate with the induction of bladder cancer by these agents, we assume that adducts other than methylpurines are responsible, although it is known that methylation alone is sufficient to transform bladder epithelium of rats (Hicks, 1980). We agree with the hypothesis put forward by Okada (1984) that alternating tissue specificity depending on even- or odd-chain length can best be explained by the Knoop type of  $\beta$ -oxidation which, in the case of even-numbered nitrosomethylalkylamines would eventually

yield *N*-nitrosocarboxypropylmethylamine, a potent bladder carcinogen in rats. If this were true, one might expect bladder tumours also after administration of *N*-nitrosobutylmethylamine (C4) and *N*-nitrosohexylmethylamine (C6), which should similarly be oxidized to form *N*-nitrosocarboxypropylmethylamine. These compounds do not usually induce bladder tumours (Druckrey *et al.*, 1967; Okada, 1984), but this may be due to the very rapid and fatal induction of oesophageal carcinomas.

# IMMUNOCYTOCHEMICAL STUDIES ON THE FORMATION AND REPAIR OF *O*<sup>6</sup>-ALKYLGUANINE IN RAT TISSUES

E. Scherer, A.A.J. Jenner & L. Den Engelse

*The Netherlands Cancer Institute, Division of Chemical Carcinogenesis,  
Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands*

The immunocytochemical visualization of the carcinogen-DNA adducts *O*<sup>6</sup>-ethylguanine (*O*<sup>6</sup>-etGua) and *O*<sup>6</sup>-methylguanine (*O*<sup>6</sup>-meGua) in histological sections of a large series of rat tissues, including liver, pancreas, testis and oesophagus, is described. In the liver of rats treated with *N*-ethyl-*N*-nitrosourea (ENU), *N*-nitrosodiethylamine (NDEA) or *N*-nitrosodimethylamine (NDMA), large differences were found in the formation and/or repair of *O*<sup>6</sup>-alkylGua between different cell types, between hepatocytes of different localization, and also between normal and precancerous hepatocytes. Heterogeneity of *O*<sup>6</sup>-etGua formation and repair was also found in pancreatic tissue of rats treated with ENU. *O*<sup>6</sup>-etGua was relatively persistent in nonparenchymal liver cells, pancreatic islet cells, spermatogonia, renal glomeruli and smooth-muscle cells. Our aim is to extend the immunocytochemical analysis to other DNA adducts, and ultimately to human tissues.

Detection of carcinogen-DNA adducts at the level of the individual cell became possible owing to the recent development of immunocytochemical methods using highly specific antibodies (Menkveld *et al.*, 1985). The sensitivity of the method is at present better than 10<sup>4</sup> *O*<sup>6</sup>-etGua residues per diploid genome of about 10<sup>10</sup> nucleotides. The recently improved methodology prevents loss of DNA from cut nuclei and results in good preservation of cytological detail. This new technology is currently being applied to study carcinogen-target cell interaction and the repair capabilities of relevant cell types during the successive stages of the carcinogenic process.

## **Damage of DNA in different cell types of the liver after treatment with NDEA, NDMA and ENU**

Formation and repair of *O*<sup>6</sup>-etGua in rat liver has been studied after treatment with NDEA and NDMA, both of which require enzymatic activation, and also with the directly-acting carcinogen ENU. Five hours after administration of a single dose of 12.5, 25 or 50 mg/kg NDEA, dose-dependent staining of hepatocyte nuclei was observed. The staining pattern was highly heterogeneous: only nuclei of centrilobular hepatocytes and sinusoidal cells were stained, whereas nuclei of hepatocytes, sinusoidal and bile-duct cells in the periportal regions of the liver lobule had no detectable amounts of *O*<sup>6</sup>-etGua.

Owing to its cross-reactivity with *O*<sup>6</sup>-meGua, rabbit antiserum raised against *O*<sup>6</sup>-etGua could also be used to visualize NDMA-induced (20 mg/kg) *O*<sup>6</sup>-meGua in rat liver sections. The pattern was similar to that observed with NDEA, except that the nonparenchymal

nuclei were stained much more intensely. After a single dose of ENU (140 mg/kg), the staining pattern was more homogeneous: nuclei of all cell types contained *O*<sup>6</sup>-etGua. After 3 h, most of the stained nuclei were of nonparenchymal cells, and only hepatocyte nuclei of the midzonal area of the liver lobule stained for *O*<sup>6</sup>-etGua. After 6 h, hepatocellular staining had increased all over the liver lobule, but was still most prominent in the midzonal area. Since the availability of ENU should be similar for all liver cell types, this distribution suggests that rapid *O*<sup>6</sup>-alkylGua repair capacity is the main determinant of the *O*<sup>6</sup>-etGua-specific staining pattern. By 24 h after ENU treatment ( $2 \times 100$  mg/kg), *O*<sup>6</sup>-etGua had largely been removed from hepatocyte nuclei; only cells of the midzonal area remained slightly stained. Nuclei of nonparenchymal cells still contained high amounts of *O*<sup>6</sup>-etGua, consistent with their low capacity for *O*<sup>6</sup>-etGua repair (Bedell *et al.*, 1982).

The accumulation of *O*<sup>6</sup>-alkylGua was investigated 6 h after five daily doses of 5 mg/kg NDMA or 20 mg/kg NDEA or after five doses of 50 mg/kg ENU given at 6-h intervals. After NDEA treatment, the staining of hepatocyte nuclei was much more homogeneous (periportal hepatocytes also stained) than after a single application, indicating that at least some *O*<sup>6</sup>-etGua persisted in hepatocytes at all localizations (Fig. 1); nonhepatocyte nuclei were poorly stained. After NDMA treatment, the centrilobular endothelial and Kupffer cell nuclei were stained most obviously; staining of hepatocyte nuclei was less intense and confined to the centrilobular area. This difference in effects between NDEA and NDMA might be related to the observation that the latter induces relatively high incidences of nonparenchymal tumours (Druckrey *et al.*, 1967). After ENU treatment, most of the *O*<sup>6</sup>-etGua was present in the nonparenchymal cells; no zonal heterogeneity was observed.

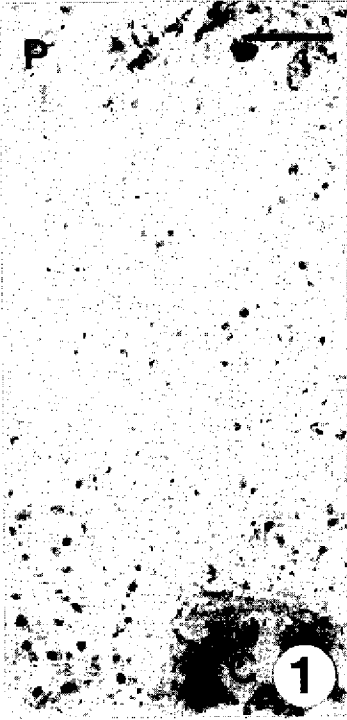
#### ***O*<sup>6</sup>-etGua in other organs**

NDEA, which can induce tumours in both the liver and the oesophagus (Druckrey *et al.*, 1967), also gave rise to strong nuclear staining of epithelial nuclei of the oesophagus (Fig. 2); this staining was detectable up to 72 h after NDEA application. In pancreas, kidney, small intestine, oesophagus and testis of ENU-treated rats ( $2 \times 100$  mg/kg), positive staining for *O*<sup>6</sup>-etGua was obtained 3 and 6 h after the last injection. In the pancreas, the staining of acinar cells largely disappeared within 24 h, indicating a considerable alkyltransferase activity. In contrast, nuclei of cells of the islets of Langerhans showed little decrease in *O*<sup>6</sup>-etGua staining between 6 and 24 h, indicating that *O*<sup>6</sup>-etGua cannot be repaired efficiently by these endocrine cells. Whereas epithelial cells of the villi from large and small intestines lost most of their *O*<sup>6</sup>-etGua within 24 h, *O*<sup>6</sup>-etGua was relatively persistent in smooth-muscle cells, spermatogonia, renal glomeruli and cells of the adrenal gland.

#### **Modification of DNA in precancerous foci of rat liver by NDEA and ENU**

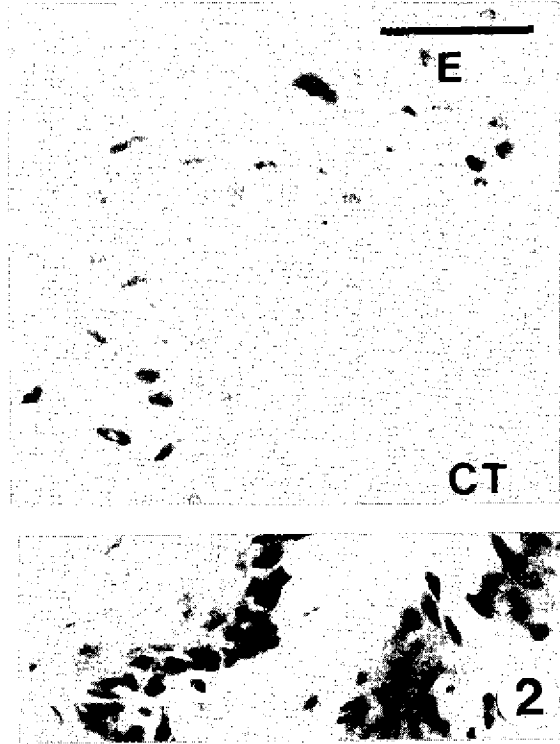
The capacity of precancerous, initiated cells to metabolize carcinogens to reactive, toxic species and to cope with carcinogen-induced (DNA) damage is an important factor during the promotion phase of neoplastic evolution (Scherer, 1984). To investigate the metabolic potential and repair of the DNA damage in precancerous liver foci, a challenging dose of NDEA or ENU was given to rats carrying such foci 3 to 24 h before sacrifice. Figure 3 shows that nuclei of 2-acetylaminofluorene-selected precancerous foci (Solt-Farber protocol) contained no or only marginal amounts of *O*<sup>6</sup>-etGua after NDEA treatment. Similar results were obtained for unselected foci. We conclude that the absence of *O*<sup>6</sup>-etGua is not due to an exceptionally high *O*<sup>6</sup>-alkyltransferase activity but reflects decreased ability

**Fig. 1.** Localization of *O*<sup>6</sup>etGua in hepatocyte nuclei after five daily doses (20 mg/kg) of NDEA



Note the homogeneous staining. Bar, 100  $\mu$ m. Taken from cryostat sections stained by the double peroxidase-antiperoxidase technique according to Menkveld *et al.* (1985). The specific antiserum used was raised in rabbits against *O*<sup>6</sup>-ethylguanosine coupled to bovine serum albumin.

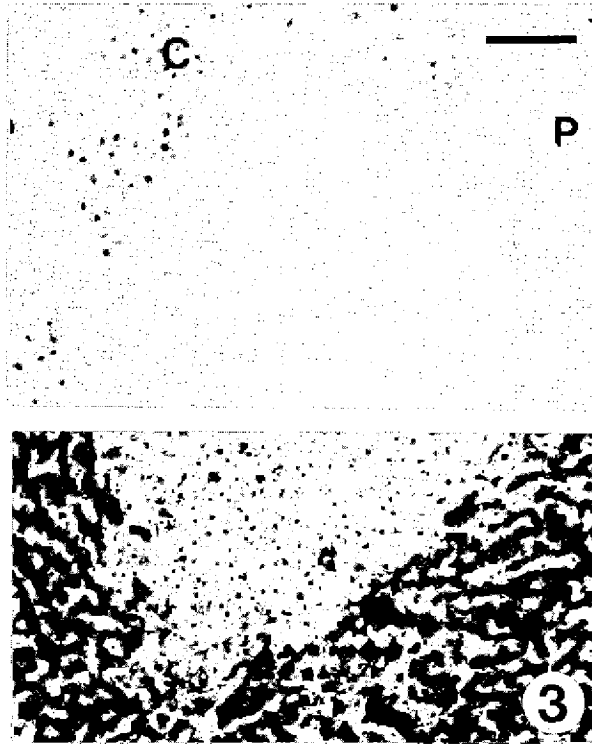
**Fig. 2.** *O*<sup>6</sup>-etGua-specific staining (top) of oesophagus epithelial nuclei 6 h after a single dose of NDEA



Nuclei of underlying connective tissue and smooth-muscle cells are unstained (compare haematoxylin and eosin-stained parallel section, bottom). E, epithelial cells; CT, connective tissue. Bar, 40  $\mu$ m. Taken from cryostat sections stained by the double peroxidase-antiperoxidase technique according to Menkveld *et al.* (1985). The specific antiserum used was raised in rabbits against *O*<sup>6</sup>-ethylguanosine coupled to bovine serum albumin.

to activate NDEA metabolically, since studies with ENU-treated rats showed that *O*<sup>6</sup>-etGua repair capacity is about the same for focus cells and surrounding normal hepatocytes. 2-Acetylaminofluorene-selected foci were also deficient in nuclear staining for guanine-aminofluorene and/or guanine-acetylaminofluorene modifications, indicating absence of the relevant activating enzymes.

**Fig. 3. Precancerous foci of rat liver selected by 2-acetylaminofluorene remain unstained for *O*<sup>6</sup>-etGua (top) after a challenging dose of NDEA (50 mg/kg)**



The focus is recognized by deficient ATPase staining (bottom). P, portal tract; C, central vein; Bar, 100  $\mu$ m. Taken from cryostat sections stained by the double peroxidase-antiperoxidase technique according to Menkveld *et al.* (1985). The specific antiserum used was raised in rabbits against *O*<sup>6</sup>-ethylguanosine coupled to bovine serum albumin.

### Concluding remarks

A semiquantitative immunocytochemical analysis of the formation and stability of DNA damage induced by *N*-nitroso compounds has been performed. With the appropriate antibodies, this technology has been found to be suitable for other DNA lesions as well. Antibodies have been raised in our laboratory for deoxyguanosine-8-acetylaminofluorene or deoxyguanosine-8-aminofluorene, cisplatin-DNA and benzo[*a*]pyrene-DNA (Dr E. Kriek and Dr L. Den Engelse). The sensitive detection and quantification of carcinogen-DNA adducts in controlled scanning microscopy is currently under investigation (in collaboration with Dr J.S. Ploem, Leiden University).

## MOLECULAR DOSIMETRY OF DNA ALKYLATION DURING CHRONIC EXPOSURE TO CARCINOGENS

J.A. Swenberg, J.A. Boucheron, F.H. Deal, F.C. Richardson & L.A. Tyeryar

*Department of Biochemical Toxicology and Pathobiology, Chemical Industry  
Institute of Toxicology, Research Triangle Park, NC 27709, USA*

Incorporation of the molecular dosimetry of DNA adducts is being proposed as a means for placing quantitative risk assessment on a stronger scientific basis. While this is likely to be an improvement over straight mathematical extrapolation, we believe that a more holistic approach that incorporates even more biology is needed. Therefore, we have begun to quantify the dose-response relationships for *N*-nitrosodiethylamine (NDEA)-induced hepatocarcinogenesis by characterizing the major promutagenic DNA adduct, *O*<sup>4</sup>-ethyldeoxythymidine (*O*<sup>4</sup>-etdT); hepatocyte proliferation; and hepatocyte initiation in rats continually exposed to drinking-water containing NDEA. The results show that *O*<sup>4</sup>-etdT accumulates to apparent steady-state concentrations that are proportional to dose at all but the highest exposures, at which less than linear amounts are found. This appears to be due to excessive cytotoxicity, since hepatocyte proliferation is markedly increased at high but not at low exposures. Hepatocyte initiation, as determined by the presence of  $\gamma$ -glutamyl transferase-positive foci, appears to have limitations in sensitivity that preclude investigations at low exposures. These methods may provide valuable insight into mechanisms of hepatocarcinogenesis at moderate exposures. Collecting these data should help to identify endpoints that may be relevant for human risk assessment.

Current methods employed in quantitative risk assessment utilize the dose-response relationship for tumours induced in experimental animals and estimates of human exposure in order to extrapolate parameters of human risk for cancer mathematically. Such models do not utilize data on differences between high and low doses in absorption, distribution, biotransformation, cytotoxicity, cell proliferation or DNA repair. Therefore, there has been considerable interest in developing scientifically based models for quantitative risk assessment that utilize measures of target site dosimetry and cell proliferation.

A review of the literature revealed a paucity of data suitable for developing mechanistically-oriented methods for quantitative risk assessment. Cancer bioassay data on large numbers of rodents are available for NDEA (Peto *et al.*, 1984) and 2-acetylaminofluorene (Staffa & Mehlman, 1979); however, only the data on NDEA cover doses ranging over several orders of magnitude. Previous studies from this laboratory have quantified the promutagenic DNA adducts *O*<sup>6</sup>-alkylguanine and *O*<sup>4</sup>-alkyldeoxythymidine in target and nontarget cells during continuous exposure to methylating and ethylating hepatocarcinogens (Bedell *et al.*, 1982; Swenberg *et al.*, 1984; Richardson *et al.*, 1985; Belinsky *et al.*, 1986a; Dyroff *et al.*, 1986; Richardson *et al.*, 1986). These studies demonstrated that *O*<sup>4</sup>-etdT accumulates in rat hepatocytes to concentrations at least 50-fold higher than *O*<sup>6</sup>-ethyldeoxyguanosine. Four-week-old rats were found to be much more sensitive to



hepatocyte initiation and hepatocarcinogenesis than were eight-week-old rats, even though  $O^4$ -etdT concentrations were similar. This increase in sensitivity was due primarily to increased cell proliferation in the younger rats.

Because of the large data base on carcinogenesis and the capacity to quantify DNA adducts and other biological endpoints, NDEA appeared to represent the compound of choice for developing a data set suitable for mechanistically-orientated quantitative risk assessment. The objective of this research was to quantify the molecular dosimetry of  $O^4$ -etdT, the extent of hepatocyte proliferation and the number of initiated hepatocytes in male Fischer 344 rats exposed to drinking-water containing 0.4, 1, 4, 10, 40 or 100 ppm NDEA for 1, 4, 7, 14, 28, 49 or 70 days.

### **Molecular dosimetry of $O^4$ -etdT**

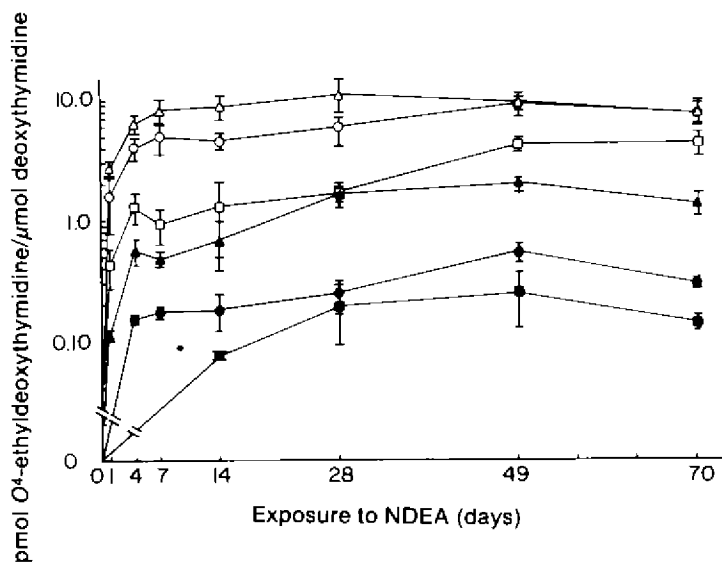
Male Fischer 344 rats were exposed to drinking-water containing NDEA beginning at six weeks of age, and NDEA consumption was monitored throughout the study. It decreased with time in all groups, but was proportional to dose from 0.4 to 40 ppm for the first five weeks and from 0.4 to 10 ppm for the entire experiment. Rats exposed to 100 ppm NDEA had decreased water consumption throughout the experiment, and rats receiving 40 ppm NDEA had lower consumption during the last five weeks of exposure.

The presence of  $O^4$ -etdT in rat liver was measured using radioimmunoassay, as described previously (Dyroff *et al.*, 1986).  $O^4$ -etdT accumulated in a dose- and time-responsive manner (Fig. 1). During the first week of exposure,  $O^4$ -etdT rapidly increased, reaching 32, 24, 21, 54 and 74% of the maximum concentration with 1, 4, 10, 40 and 100 ppm NDEA, respectively. Thus,  $O^4$ -etdT concentrations approached their maxima more rapidly with exposure to 40 and 100 ppm NDEA. Adduct concentrations were below the limit of detection for animals receiving 0.4 ppm NDEA for less than 14 days and for those receiving 1 ppm on day 1. It was difficult to establish clearly when or if  $O^4$ -etdT concentrations achieved a steady-state, due to decreasing consumption rates over time. However, apparent steady-state concentrations were obtained between 4 and 28 days and were concentration-dependent over the entire dose range. When  $O^4$ -etdT concentrations at 49 days of exposure were examined for linearity with respect to dose, DNA adduct concentrations were found to increase linearly from 0.4 to 40 ppm NDEA, but were less than linear at exposures of 100 ppm.

### **Dose-response relationship for NDEA and hepatocyte proliferation**

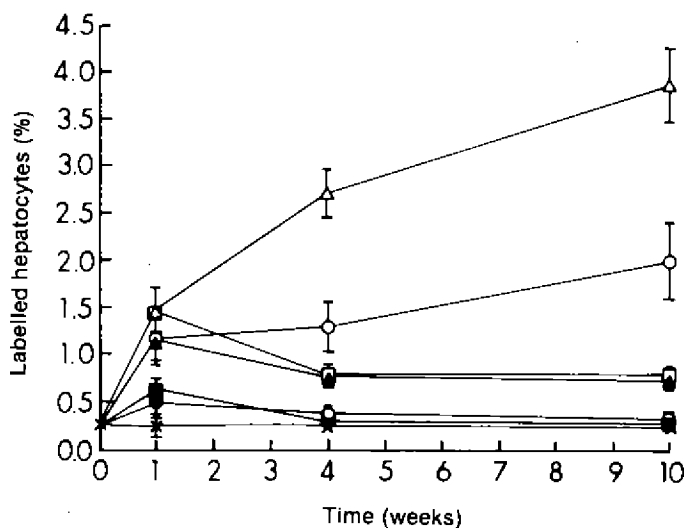
Rats were exposed to NDEA for 1-, 4- or 10-week regimens similar to those described for the studies with  $O^4$ -etdT. The animals were injected intraperitoneally with  $^3$ H-thymidine 2 h before they were killed, sections of liver were processed for autoradiography and the labelling index of hepatocytes was determined. The dose-response relationship for NDEA and hepatocyte proliferation in the left lobe of the liver is shown in Figure 2. A marked and progressive increase in replication that reached 1500% of control was induced by exposure to 100 ppm NDEA. Likewise, a 500-800% increase was present in hepatocytes of rats exposed to 40 ppm NDEA. Exposure to 10 or 4 ppm NDEA resulted in a 300% increase in hepatocyte replication at 4 and 10 weeks, whereas the numbers of labelled hepatocytes from rats exposed to 1 and 0.4 ppm were not significantly elevated over those in controls. Similar trends were observed for cell proliferation in the right median and right anterior lobes, although the extent was lower. Thus, the extent of cell proliferation was concentration dependent. These differences may alter the number of promutagenic DNA adducts that mispair prior to repair and may enhance the growth of initiated cells — processes that could affect the extent of initiation and the time to tumour.

**Fig. 1.** Accumulation of  $O^6$ -etdT during continuous exposure of rats to 0.4 (■), 1 (●), 4 (▲), 10 (□), 40 (○) or 100 (△) ppm NDEA



Note the log scale of the y axis; the bars represent  $\pm$  SE for 3-4 rats.

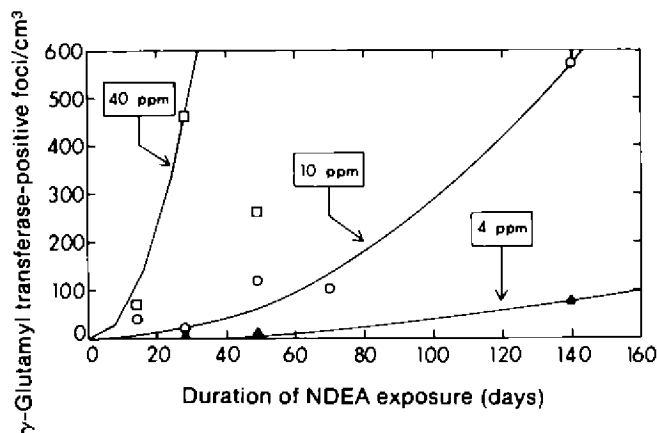
**Fig. 2.** Hepatocyte replication in the left lobe of livers of rats exposed to 0 (×), 0.4 (■), 1 (●), 4 (▲), 10 (□), 40 (○) or 100 (△) ppm NDEA in drinking-water for 1, 4 or 10 weeks



### Quantitative studies on hepatocyte initiation

Rats were exposed to similar regimens of NDEA for quantitative studies on hepatocyte initiation. Following exposure, rats were placed on a modified Cayama-Farber growth selection regimen (Richardson *et al.*, 1986), and  $\gamma$ -glutamyl transferase-positive foci were quantified as an index of hepatocyte initiation using stereological methods. The number of foci in the left hepatic lobe increased with NDEA concentration and with the length of exposure (Fig. 3). This method was not sensitive enough to demonstrate increases in hepatocyte initiation at concentrations of less than 4 ppm NDEA; and long exposures (i.e., 20 weeks) to 4 ppm NDEA were required to obtain numbers of foci that were easily quantified. Visual and mathematical analysis of the data indicated that, over the concentrations examined, foci induction was dependent on the (concentration  $\times$  time) product, rather than on the rate of NDEA consumption. Similar phenomena were observed in the median and right anterior hepatic lobes, although the quantitative relationships between foci and (concentration  $\times$  time) were different (data not shown).

**Fig. 3. Induction of  $\gamma$ -glutamyl transferase-positive foci by exposure to 4 ( $\blacktriangle$ ), 10 ( $\square$ ) or 40 (o) ppm NDEA for up to 20 weeks**



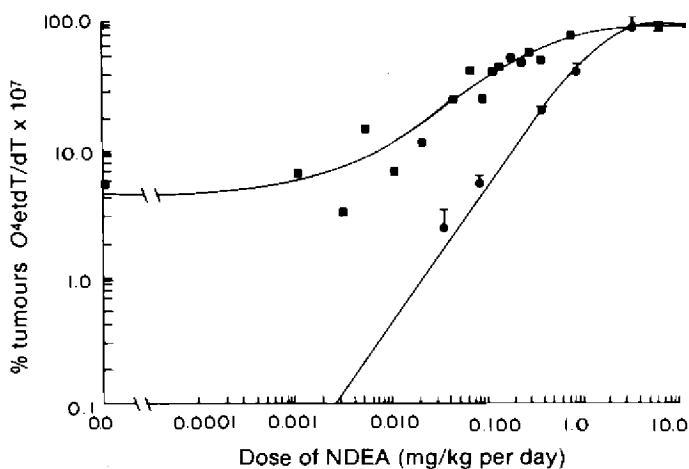
### Relevance to human cancer

Many assumptions must be incorporated in order to extrapolate data on DNA adducts, cell proliferation and carcinogenesis from rats to humans. Presently, these assumptions remain untested. The main objective of the present studies was to develop a data set suitable for testing mechanistic parameters in rats. Appropriate data can then begin to be obtained from humans, so that methods that incorporate appropriate biology for high- to low-dose extrapolation can be developed.

Important considerations include the relationship between DNA adducts and carcinogenesis. Since the design of the present experiments was similar to that of the large cancer bioassay on NDEA (Peto *et al.*, 1984), a comparison of the data has been undertaken. The incidence of hepatic neoplasia in the bioassay has been plotted for comparison with the concentrations of  $O^6$ -etdT at 49 days (Fig. 4). The dose range over which  $O^6$ -etdT was measured corresponded to the higher NDEA concentrations of the tumour study. Determination of  $O^6$ -etdT concentrations at lower exposures will require even more sensitive methods. Since there appear to be concentrations of NDEA that do not produce increases over background in tumour incidence, it is likely that factors influencing the biology of these endpoints change with decreasing concentrations of NDEA. Probable factors include the relationship between tumour formation and  $O^6$ -etdT, and the relationship between NDEA dose and  $O^6$ -etdT. Preliminary data suggest that a saturable repair system may exist that is more efficient at even lower exposures; in contrast, cell

proliferation will affect responses primarily at higher concentrations. Each of these relationships must be studied further to gain a better understanding of its role in rodent carcinogenesis, and only then will it be possible to begin testing similar endpoints and assumptions in humans. Nevertheless, the methods developed and data collected should assist in more critically defining which are the appropriate human parameters to measure in order to validate new methods for extrapolation of risk from rodents to humans, and from high to low doses.

**Fig. 4.**  $O^6$ -etdT concentrations in livers of male Fischer 344 rats after 49 days of exposure to NDEA (●) and liver tumour incidences in Colworth rats (Peto *et al.*, 1984) after lifetime exposure to NDEA (■)



To aid in visualization of the data, the adduct concentrations were fitted with a Weibull distribution function that extended through zero, and the tumour incidences were regressed against dose of NDEA using a generalized rectangular hyperbola.

# LONG-TERM PERSISTENCE OF NITROSAMINE-INDUCED STRUCTURAL DAMAGE TO HETEROCHROMATIC DNA

B.W. Stewart & E.J. Ward

*Childrens Leukaemia and Cancer Research Unit, Prince of Wales Childrens Hospital, Randwick 2031, and School of Pathology, University of New South Wales, Kensington 2033, NSW, Australia*

Different levels of damage and repair to eu- and heterochromatic DNA from the livers of rats receiving a dose of 10 mg/kg *N*-nitrosodimethylamine (NDMA) were apparent. Preincorporated <sup>3</sup>H-thymidine was lost rapidly from euchromatic DNA but persisted in the heterochromatic fraction. Persistent damage, determined as single-stranded regions binding to benzoylated DEAE-cellulose (BD-cellulose), was evident in heterochromatic DNA for up to three months. By subjecting rats treated with NDMA to partial hepatectomy, generation of single-stranded regions in the newly synthesized heterochromatic DNA could be demonstrated. Such structural defects were evident when hepatectomy was performed two months after administration of the carcinogen. These findings indicate that structural damage to nontranscribed DNA is one of the most persistent molecular lesions following exposure to nitrosamines.

Most work on nitrosamine-induced DNA repair has concerned monitoring the concentration of alkylated bases in DNA (Preussmann & Stewart, 1984), a parameter of the first stage of DNA repair. The distribution of carcinogen adducts within subfractions of DNA generated by digestion of chromatin has provided evidence of heterogeneous repair processes (for references see Baranyi-Furlong & Goodman, 1984). However, in terms of chromatin, evidence concerning intermediate and late stages of DNA repair is lacking. Information regarding these processes necessitates structural analysis of DNA.

Most studies of chromatin-carcinogen interrelationships have involved exhaustive, usually DNase I-mediated, degradation of chromatin to the single nucleosome level. However, micrococcal nuclease has become the enzyme of choice in the study of transcriptionally active chromatin, because at low levels it does not overly degrade DNA (Tata & Baker, 1978).

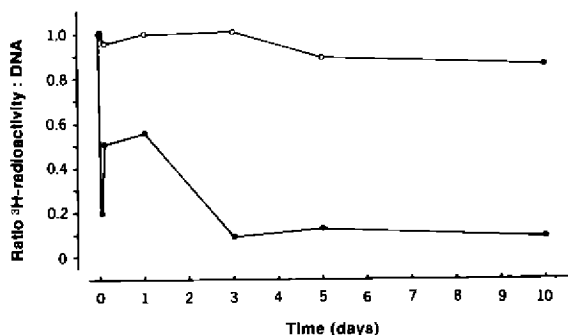
## Loss of radioactivity from euchromatic DNA

Using female Wistar rats, hepatic DNA was radiolabelled with <sup>3</sup>H-thymidine two weeks before administration of a single intraperitoneal injection of NDMA at a non-necrotizing dose of 10 mg/kg body weight. Isolated nuclei were digested and fractionated under the conditions described by Tata and Baker (1978). Following phenol extraction of either whole liver homogenate or nuclease fractionated eu- and heterochromatin, carcinogen-modified DNA was examined for structural change by either stepwise or caffeine gradient elution from BD-cellulose (Ward *et al.*, 1985).

Following injection of animals with NDMA, stepwise chromatography resulted in a higher proportion of sheared rat liver DNA being bound to BD-cellulose in the presence of 1.0 M sodium chloride. The amount of DNA thus exhibiting single-stranded character rapidly declined within 48 h and was not detectable after ten days. These DNA preparations were subjected to caffeine-gradient elution from BD-cellulose; chromatograms of DNA isolated 24 h after injection of NDMA were marginally different from those of the control, while by five days no consistent treatment-related effect could be discerned. Such transient structural change immediately following NDMA treatment is attributable to asynchrony between the initial and final stages of DNA repair (Stewart, 1981; Stewart *et al.*, 1985).

Following incubation of purified nuclei with micrococcal nuclease, 10-12% of the total labelled nuclear DNA was categorized as euchromatin as a result of solubilization using 1.0 unit of enzyme/5 mg nuclear DNA/ml after 9 min. Administration of NDMA to rats differentially affected the amount of radioactivity in the eu- and heterochromatin fractions. Within 48 h of NDMA injection, there was a marked fall in the specific radioactivity of solubilized DNA. No NDMA-induced change was observed in the specific radioactivity of DNA which sedimented after digestion (Fig. 1). The implication that loss of preincorporated radiolabelled thymidine is caused by preferential repair of transcribed DNA is consistent with observations made by Bodell (1977). Conversely, the failure of NDMA treatment to affect the specific radioactivity of heterochromatin is consistent with numerous reports of low adduct concentrations in nuclease-resistant DNA fractionated as chromatin (Berkowitz & Silk, 1981; Schwartz *et al.*, 1981).

**Fig. 1. Relative loss of preincorporated  $^3\text{H}$ -thymidine from euchromatin (●) and heterochromatin (○) following injection of rats with 10 mg/kg body weight NDMA**



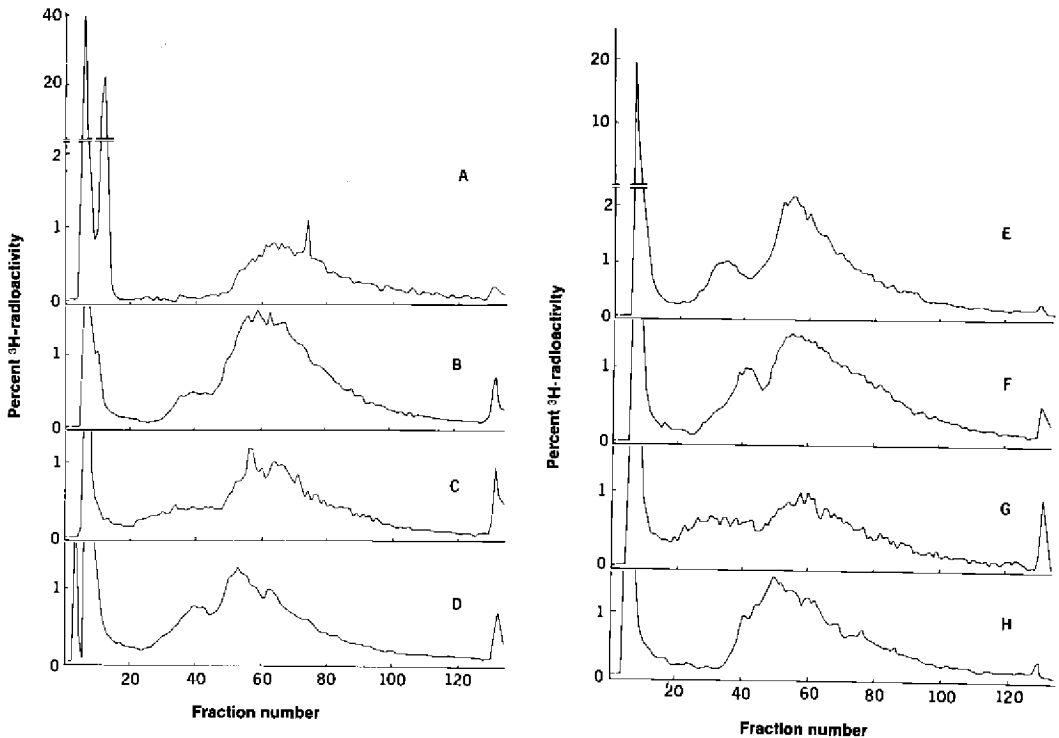
At times up to ten days after NDMA treatment, isolated liver nuclei were fractionated into eu- and heterochromatin by mild micrococcal nuclease digestion. After extraction of DNA from the respective fractions, its specific activity was determined by absorbance and scintillation counting. This ratio was normalized to 1.0 for the respective control preparations, relative to which the nitrosamine-induced changes are expressed.

#### Structural damage in heterochromatin DNA

In chromatograms of heterochromatin DNA from control rats, the principal feature was a broad peak (fractions 50-80), indicating DNA binding with the affinity of single-stranded DNA from 1000 to 5600 bases. This feature was characteristic of nuclei isolation. In immediate response to NDMA treatment, an increased amount of heterochromatin DNA was eluted at low caffeine concentrations (fractions 30-50) in a similar manner to that which was noted in unfractionated DNA preparations 1-4 h after administration of the carcinogen. Chromatograms of DNA from heterochromatin isolated five days to one month after NDMA treatment were characterized by the generation, and subsequent decline, of a peak in fractions

20-50. By three months, the chromatogram could not readily be distinguished from that of the control (Fig. 2).

**Fig. 2. Effect of treatment with NDMA on the structure of DNA extracted from rat-liver heterochromatin as determined by caffeine-gradient elution from BD-cellulose**



DNA was adsorbed to the column in 0.3 M sodium chloride (fractions 1-5, 10 ml) and, after elution of double-stranded DNA in 1.0 M sodium chloride (6-10, 10 ml), DNA containing single-stranded regions was eluted using a biphasic linear 0-0.8% caffeine gradient (11-129, 240 ml; Ward *et al.*, 1985), the final wash being done with 2% caffeine (10 ml). DNA from control rats was analysed (A) as well as that from NDMA-treated animals killed 1 h (B), 4 h (C), 24 h (D), five days (E), ten days (F), one month (G) and three months (H) after injection of the carcinogen. Each chromatogram is typical of at least two results, and radioactivity per chromatogram was  $1.4 \times 10^4$  dpm.

Euchromatic control profiles were similar to their heterochromatin counterparts from animals that did not receive NDMA. In comparison, some slight change was apparent in chromatograms generated from micrococcal-solubilized DNA, within 4 h of NDMA administration. However, similar studies undertaken three days after carcinogen treatment resulted in chromatograms similar to those of controls.

The effect of parental-strand lesions on daughter-strand synthesis was assessed by subjecting animals to partial hepatectomy at intervals after administration of NDMA. These experiments involved radiolabelling of DNA 24 h after hepatectomy and sacrifice a further 24 h later. BD-cellulose chromatograms of newly-replicated heterochromatin DNA from rats exposed up to two months previously to NDMA exhibited two broad peaks. In common with preparations from saline-treated controls, radioactivity was recovered in a broad peak from fractions 50-80 as a consequence of endogenous nuclease(s) (Ward *et al.*, 1985). However, NDMA treatment caused recovery of DNA in a sharp peak earlier in the gradient. Although most conspicuous when hepatectomy was performed ten days after NDMA injection, this effect was still evident when the interval between carcinogen treatment and hepatectomy was extended to two months. It is presumably due to replication of DNA on an alkylation-damaged template.

#### **Significance of heritable structural damage**

The present findings suggest the persistence of minor lesions in nontranscribed DNA. Examination by Chow and Fraser (1983) of the endo-exonuclease of *Neurospora crassa* suggests that, in rat liver nuclei, repair sites may be preferential substrates for nuclease(s). Alkylated bases could give rise to single-stranded gaps after nuclease attack of distorted DNA (Columbano *et al.*, 1980). Incomplete filling of repair gaps or failure of ligation could cause lesions (gaps of approximately five nucleotides or less) which would not bind to BD-cellulose but could give rise to binding after nuclease attack. If both alkylated bases and single-stranded regions *per se* constitute damage in nitrosamine-exposed DNA, damage in DNA synthesized subsequent to carcinogen exposure would almost certainly be single-stranded regions or other structural lesions. Since the secondary structure of DNA appears to play a critical role in gene expression (Elgin, 1981), it is not difficult to envisage relationships between the present findings and the role of cell replication in carcinogenesis.



## **N-NITROSO-N-METHYLANILINE: POSSIBLE MODE OF DNA MODIFICATION**

**S.R. Koepke, M.B. Kroeger-Koepke & C.J. Michejda**

*Laboratory of Chemical and Physical Carcinogenesis, LBI-Basic Research  
Program, NCI - Frederick Cancer Research Facility, Frederick, MD 21701, USA*

*N*-Nitroso-*N*-methylaniline (NMA) has been shown to be an oesophageal carcinogen in a number of rat strains (Druckrey *et al.*, 1967; Napalkov & Pozharissiki, 1969; Goodall *et al.*, 1970; Kroeger-Koepke *et al.*, 1983). A large number of *N*-nitrosomethylalkylamines have also been shown to produce tumours in that organ in rats (Druckrey *et al.*, 1967). A common feature of these nitrosamines is that they can be metabolized to an alkylating intermediate —specifically, a methylating agent (Heath, 1962; Magee & Barnes, 1967; Miller, 1970). The metabolism of NMA, however, is unique in that it appears that the benzenediazonium ion (BDI) is the electrophilic intermediate (Michejda *et al.*, 1982); no methyl diazonium ion appears to be formed. BDI has been shown to be a potent mutagen in the Ames' assay (Gold & Salmasi, 1982). Using the methods of Herron and Shank (1979), we failed to detect any induced DNA methylation *in vivo*. In addition, it was demonstrated that neither hepatic DNA nor RNA was alkylated with either the phenyl ring or methyl group in rats using radiolabelled NMA. Other reactions such as denitrosation have been shown to occur (Gold, personal communication). Taken collectively, these data leave the question of mode of activation of NMA largely unanswered.

### **Metabolism of NMA *in vitro***

A 9000 × *g* supernatant fraction from uninduced male Fischer 344 rats was used for in-vitro metabolism studies of [<sup>14</sup>C]NMA. The formation of the postulated BDI was assayed by detection of the corresponding azo dye coupling product following the addition of phenol as the trapping agent to the medium. The formation of *p*-hydroxyazobenzene from [<sup>14</sup>C]NMA, when the reaction was carried out in the presence of phenol and terminated after 1 h by the addition of trichloroacetic acid, was determined to account for 4.7% of the radioactivity in the initial substrate. Analysis of the disappearance of NMA showed that 5.9% of the substrate had been consumed. The blank control reactions (without NMA) showed no detectable formation of *p*-hydroxyazobenzene.

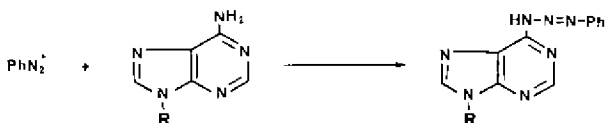
The results of this study clearly demonstrate that the BDI was stable enough in in-vitro reactions of NMA to be trapped by coupling with phenol. Of the 5.9% total metabolism of NMA, we can account for 71.5% of the diazonium ion as the *p*-hydroxyazobenzene coupling product. In the absence of phenol, the diazonium ion is probably trapped by other aromatic substances present in the mix. Other reactions, such as denitrosation, may account for the remainder of the metabolism. We determined previously that no ring hydroxylation of NMA occurred *in vitro* (Kroeger-Koepke, unpublished data).

It is clear that BDI is a major intermediate in the metabolism of NMA, as predicted by the  $\alpha$ -hydroxylation hypothesis. Furthermore, it is likely that coupling reactions with aromatic substances present in the medium (e.g., nucleic acids) account for many of the other metabolic breakdown products.

### Reactions of BDI with DNA

It has been shown (Chin *et al.*, 1981) that various substituted BDI are capable of reacting with adenine, adenosine and adenylic acid to form *N*-6 triazenes with the exocyclic amino group (Scheme 1). These triazenes can then decompose to the 8-aryl species *via* a free-radical pathway. In a continuation of these studies (Hung & Stock, 1982), the reactions of BDI with guanine, guanosine and guanylic acid were also examined. Only in the case of guanylic acid were the authors able to find any evidence for a low yield of the *N*-2 triazene.

#### Scheme 1. Formation of triazenes from exocyclic amino groups



cellulose and then reacted with the nucleic acid. A study of the nature of the linkage has been made utilizing nucleotide homopolymers (Noyes & Stark, 1975). The data listed in Table I seem to suggest that poly(C) and poly(A) do not react well with the cellulose. However, if we interpret the previous work with BDI and adenine, it seems quite likely that reaction does occur with these polymers, but the linkage may be *via* a relatively unstable triazene. In light of this interpretation, a possible pathway for the mode of action of NMA can be postulated.

**Table 1. Coupling of nucleotide homopolymers to diazo cellulose**

Polymer	% Coupled
Poly(U)	66
Poly(dT)	20
Poly(C)	<5
Poly(G)	41
Poly(A)	<5

mutagenesis by nitrous acid. A similar deamination pathway has been shown to occur for cytosine, adenine and guanine with this mutagen (Becker *et al.*, 1964; Shapiro & Klein, 1966).

The triazene formed from adenylic acid has been shown to decompose in only 10-20% yield to the arylated base (Chin *et al.*, 1981). The other 80-90% of the triazene was left unaccounted for and did not revert to any form of the starting base. Since the deaminated base was not specifically examined, it is possible that deamination of adenylic acid could have occurred.

Evidence for the reactions of DNA with diazonium ions can also be garnered from the standard method for covalently binding DNA and RNA to cellulose for use in various analytical biochemical procedures (Alwine *et al.*, 1979). Diazo-ized aryl amine is bound to the

#### Possible mechanism of reaction

If the triazenes of adenine and cytosine can be formed *in vivo* in DNA, then a tautomerization followed by decomposition would yield the corresponding diazonium ions of the bases. Although it is possible that these reactive species could then serve to cross-link the DNA, it would seem more probable that decomposition to hypoxanthine and uridine would occur. In effect, a point mutation would then be possible through a deamination reaction. This scenario is not unreasonable when one examines the literature on the mechanism of

### Studies on mode of action

In an effort to learn more about the interaction of NMA with DNA, we first examined the reaction of BDI with calf thymus DNA. The calf thymus DNA was treated with BDI hexafluorophosphate at 37°C for 30 min at pH 7.4 and then precipitated with ethanol, redissolved in distilled water, and reprecipitated with ethanol twice more to remove any unreacted BDI. (The last wash was treated with phenol to check on the efficiency of the washes.) After drying, the calf thymus DNA was dissolved in 0.1 M phosphate buffer (pH 7.4), and 20 mg phenol were added to trap any diazonium ion formed. The pH was adjusted to 3.0 with 1.0 M hydrochloric acid and the reaction shaken for 1 h at room temperature. The sample was lyophilized, suspended in methanol and filtered; the solvent was removed under vacuum; the residue was resuspended in ethyl acetate and then refiltered. The filtrate was analysed by high-performance liquid chromatography for the presence of the phenol-BDI coupling product, 4-hydroxyazobenzene. Yields of this material, as determined by ultraviolet-visible spectroscopy, ranged from 800-1400  $\mu\text{mol/mol}$  guanine.

Experiments on the in-vitro interaction of calf thymus DNA with NMA oxidized with the 9000  $\times$  g supernatant fraction from uninduced rat liver were also performed. The calf thymus DNA was isolated *via* the phenol extraction procedure (Lai *et al.*, 1979), and the isolated DNA was treated in a manner similar to that described above. The yield of hydroxyazobenzene was found to be 58  $\mu\text{mol/mol}$  guanine.

Hepatic DNA obtained from Fischer 344 treated with 226 mg/kg NMA in corn oil by gavage was also subjected to the phenol coupling procedure using  $^{14}\text{C}$ -phenol to enhance sensitivity. No labelled coupling product was detected in these experiments. However, on the basis of the data obtained *in vitro*, the amount of the coupling product would have been very small — perhaps beyond our ability to detect it. Additional in-vivo experiments will provide more definitive data.

### Conclusions

The chemical and in-vitro data support triazene formation in the reaction of DNA with BDI. To date, in-vivo experiments have been inconclusive. When relative yields (unit azo dye formation per unit BDI) are examined, it is apparent that the amount of coupling product in the reaction with metabolic activation is 10-20 times lower than that expected.

There are two possible explanations. First, BDI can react readily with protein, thus reducing its effective concentration. Second, it is also likely that even the relatively short phenol extraction procedure can catalyse the decomposition of the relatively unstable triazenes of the nucleic acid bases. We are currently exploring both possibilities.

Although our trapping experiments provide evidence for triazene formation with DNA, at the time of this writing, we have no direct evidence for the deamination process. However, earlier work with nitrite, which indicated that this reaction is a promutagenic lesion, suggests that it is likely to occur in the case of NMA.

### Acknowledgements

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**$\beta$ -OXIDIZED *N*-NITROSOALKYL CARBAMATES  
AS MODELS FOR DNA ALKYLATION BY  
*N*-NITROSOBIS(2-OXOPROPYL)AMINE  
IN SYRIAN HAMSTERS**

D.L. Nagel, R. Lewis, M. Fischer, K. Stansbury,  
K. Stepan & T.A. Lawson

*The Eppley Institute for Research in Cancer,  
University of Nebraska Medical Center, Omaha, Nebraska 68105, USA*

A single dose of *N*-nitrosobis(2-oxopropyl)amine (NDOPA) can selectively induce pancreatic-duct adenocarcinomas in Syrian hamsters. Multiple doses or a higher single dose can induce tumours of the liver and of other organs. Our earlier studies employing NDOPA systematically labelled with  $^{14}\text{C}$  in the three-carbon chain showed that hamster pancreatic DNA is almost exclusively methylated and that the sole source of the methyl group is the  $\alpha$  carbon of NDOPA. Hamster liver DNA was equally methylated and alkylated by a three-carbon chain. Current studies using generally labelled tritiated NDOPA with a very high specific activity have shown that the three-carbon alkylation is 2-hydroxypropylation. We have identified two adducts isolated from hamster liver DNA, *N*7-(2-hydroxypropyl)guanine and *O*<sup>6</sup>-(2-hydroxypropyl)guanine, which contain this group, and we have also isolated and identified *N*7-methylguanine and *O*<sup>6</sup>-methylguanine in DNA from hamster liver and pancreas.  $\beta$ -Oxidized *N*-nitrosocarbamates, ethyl *N*-nitroso-2-oxopropylcarbamate (NOPC) and ethyl *N*-nitroso-2-hydroxypropylcarbamate (NHPC), are useful models for predicting the DNA adducts observed *in vivo* following NDOPA treatment. Base-catalysed decomposition of NOPC in the presence of exogenous DNA yields five methylated purines (*N*3-, *N*7- and *O*<sup>6</sup>-methylguanines and *N*1- and *N*3-methyladenines). NHPC, a model for *N*-nitrosamines containing the 2-hydroxypropyl group, reacts with guanosine to yield *N*7- and *O*<sup>6</sup>-(2-hydroxypropyl)guanines. These results suggest that the methylation of DNA is mediated through  $\alpha$ -hydroxylation on the opposite chain of a nitrosamine containing a 2-oxopropyl or methyl group and that 2-hydroxypropylation results from  $\alpha$ -hydroxylation on the opposite chain of a nitrosamine containing a 2-hydroxypropyl group. The alternative mechanism, that of sulfation of  $\beta$ -hydroxy nitrosamines, cannot be entirely ruled out for production of 2-hydroxypropyl adducts.

A single subcutaneous dose of NDOPA selectively induces pancreatic cancer in Syrian hamsters. At a higher single dose or with multiple doses, tumours of the liver and other organs also occur (Pour *et al.*, 1978). *N*-Nitrosobis(2-hydroxypropyl)amine (NDHPA), a metabolite of NDOPA, also induces pancreatic cancer in hamsters and has been found in environmental samples; hence, it may constitute an environmental hazard (Issenberg *et al.*, 1984). Two other nitrosamine metabolites of NDOPA, which also induce pancreatic cancer

in hamsters, are *N*-nitroso(2-hydroxypropyl)(2-oxopropyl)amine (NHPOPA) and *N*-nitrosomethyl(2-oxopropyl)amine (NMOPA) (Pour *et al.*, 1981). These nitrosamines require metabolic activation to express their mutagenicity in the Ames assay and transforming ability in the hamster liver-cell-mediated assay (Langenbach *et al.*, 1980). Our current knowledge suggests that the  $\alpha$ -hydroxy derivatives are critical activation intermediates.

In earlier studies, we showed that pancreatic DNA isolated from hamsters treated with NDOPA was almost entirely methylated, whereas liver DNA from these animals was both methylated and alkylated with a larger group of unknown structure (Lawson *et al.*, 1981).

*N*-Nitrosoalkylcarbamates have been used as models to study the alkylation of dialkyl nitrosamines following  $\alpha$ -hydroxylation, since they decompose through common intermediates, alkyl diazotates. In an effort to elucidate the mechanism of activation of NDOPA, we chose to study the base-catalysed decomposition of two  $\beta$ -oxidized *N*-nitrosocarbamates, NOPC and NHPC, as possible models for the action of  $\beta$ -oxidized nitrosamines *in vivo*. NOPC was chosen as a model for nitrosamines containing the 2-oxopropyl group and NHPC as a model for several of the metabolites of NDOPA that contain the 2-hydroxypropyl group.

### Studies with NOPC

NOPC has a half-life of 45 min in aqueous solution at pH 7.4. The products of the decomposition are given in Table 1. Two of the products, diazoacetone and diazomethane, are unstable and decompose further to yield hydroxyacetone and methanol, respectively. The decomposition of diazoacetone is accelerated at low pH (<4). When NOPC was decomposed in the presence of calf thymus DNA and the DNA hydrolysed and analysed by high-performance liquid chromatography, only methylated adducts (Table 2) were detected. No reaction was detected between diazoacetone and guanosine at pH 7.4.

**Table 1. Products obtained from the base-catalysed decomposition of NOPC at pH 7.4**

Compound <sup>a</sup>	Yield <sup>b</sup> (%)
Ethanol	89.2 ± 8.9
Hydroxyacetone	44.4 ± 2.4
Methanol	38.6 ± 3.3
Acetic acid	39.0 ± 3.9
Ethyl (2-hydroxypropyl)- carbamate	11.3 ± 3.6
Diazomethane	<i>c</i>
Diazoacetone	<i>d</i>

<sup>a</sup>Products were analysed by gas chromatography and structures confirmed by gas chromatography/mass spectrometry.

<sup>b</sup>Based on NOPC

<sup>c</sup>Confirmed by trapping with *N*-ethylmaleimide

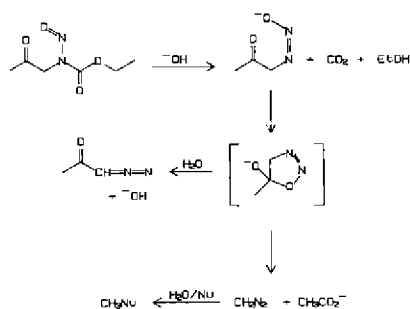
<sup>d</sup>Confirmed by trapping with *N*-ethylmaleimide and infrared spectroscopy

These observations are consistent with the mechanism of decomposition of NOPC proposed in Figure 1. NOPC decomposes initially to yield an intermediate diazohydroxide, which then cyclizes to the oxadiazoline. This intermediate then decomposes by two different routes: diazomethane and acetic acid are produced by one route and diazoacetone by the other. The distribution of the DNA methylated adducts is consistent with methylation by diazomethane. These results suggest that four nitrosamines (following  $\alpha$ -hydroxylation) may be responsible for the DNA methylation observed in hamsters following treatment with NDOPA; they are NDOPA, NHPOPA, NMOPA and *N*-nitrosomethyl(2-hydroxypropyl)amine (NMHPA).

**Table 2. Methylated adducts produced by decomposition of NOPC in the presence of calf-thymus DNA**

Base <sup>a</sup>	Retention time (min)	Methylated bases (%)
1-Methyladenine	4.9	6.4 $\pm$ 0.7
3-Methyladenine	5.5	7.0 $\pm$ 0.7
3-Methylguanine	6.3	6.8 $\pm$ 0.8
7-Methylguanine	10.8	70.4 $\pm$ 4.6
6-Methylguanine	18.8	12.1 $\pm$ 1.2

<sup>a</sup>Products were analysed by high-performance liquid chromatography following acid hydrolysis. A Whatman Partisil 9 C8 column was used with 0.2M ammonium phosphate and 8% methanol as the eluent.

**Fig. 1. Proposed mechanism of the base-catalysed decomposition of NOPC; Nu, nucleophile****Table 3. Products obtained from the hydrolysis of NHPC at pH 7.4**

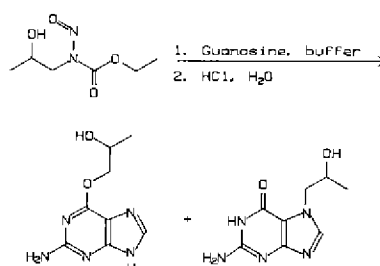
Product <sup>a</sup>	Yield <sup>b</sup> (%)
Acetone	22.5
Propylene oxide	0.7
Propanal	4.2
Ethanol	76.1
Ethyl 2-hydroxypropyl carbonate	4.8
Propylene carbonate	10.9
Propylene glycol	27.1

<sup>a</sup>Determined by gas chromatography with confirmation by gas chromatography/mass spectrometry

<sup>b</sup>Based on NHPC

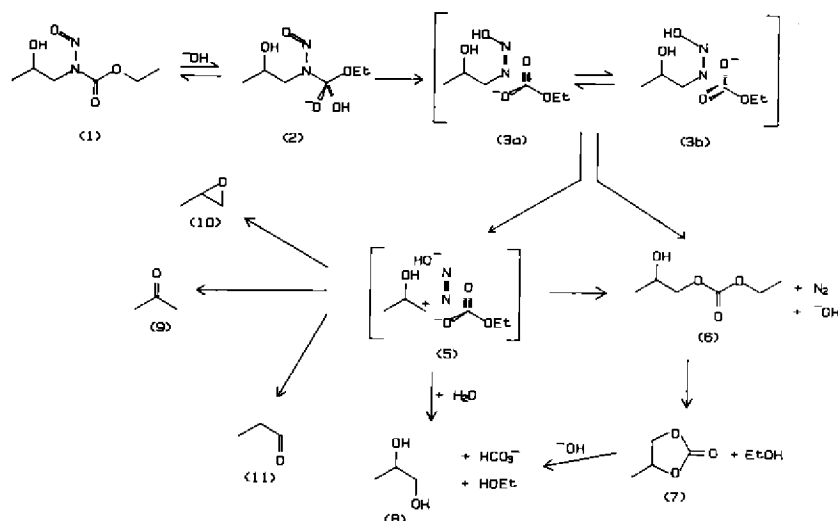
### Studies with NHPC

NHPC has a half-life of 10 min in aqueous buffer at pH 7.4. The products of the decomposition are given in Table 3. When NHPC was decomposed in the presence of guanosine, two guanine adducts were isolated and characterized following acid hydrolysis. They were identified as *N*7-(2-hydroxypropyl)guanine and *O*6-(2-hydroxypropyl)guanine (Fig. 2).

**Fig. 2. Structure of adducts produced from the reaction of guanosine and NHPC; \*, undergoes further decomposition**

These observations are consistent with the mechanism of base-catalysed decomposition of NHPC shown in Figure 3. NHPC initially decomposes to a nitrogen-separated ion triplet (5). Three of the products (propylene oxide, acetone and propionaldehyde) result from rearrangement of the hydroxypropyl carbonium ion within the nitrogen-separated ion triplet (5). Ethyl 2-hydroxypropyl carbonate, an internal return product, is unstable at physiological pH and decomposes to propylene carbonate. Above pH 8.0, propylene carbonate is hydrolysed to propylene glycol. Solvolysis of 5 can also lead to propylene carbonate. The two guanine adducts presumably result from reaction with 5 and guanosine; they were formed in approximately equal amounts.

Fig. 3. Proposed mechanism of the base-catalysed decomposition of NHPC



### In-vivo alkylation studies with Syrian hamsters and NDOPA

Syrian hamsters were treated with 5 mCi  $^3\text{H}$ -NDOPA at a dose of 10 mg/kg body weight. The animals were killed 5 h later and the pancreas and liver excised; the DNA was isolated, purified and acid hydrolysed. The hydrolysate was spiked with nonradioactive standards of methylated and hydroxypropylated purines and analysed by high-performance liquid chromatography on a reverse-phase column. The fractions that coeluted with the standards were concentrated and rechromatographed on a strong cation-exchange column. Quantification of the adducts was accomplished by scintillation counting. Four adducts were detected in hamster liver DNA —  $N^7$ -methylguanine,  $N^7$ -1-(2-hydroxypropyl)guanine,  $O^6$ -methylguanine and  $O^6$ -1-(2-hydroxypropyl)guanine. In pancreatic DNA, only the two methylated guanines were detected.

### Conclusions

These results suggest that the nitrosoalkylcarbamates containing a hydroxyl or keto group in the  $\beta$  position are useful predictors of the mode of DNA alkylation by  $\beta$ -keto nitrosamines *in vivo*. It also lends support to the hypothesis that  $\alpha$ -hydroxylation is the mechanism of activation of NDOPA.  $\alpha$ -Hydroxylation of NDOPA and NMOPA or of NHPOPA and NMHPA on the 2-hydroxypropyl side chain should lead to methylation of DNA, whereas  $\alpha$ -hydroxylation of NHPOPA on the 2-oxopropyl side chain or of NDHPA should lead to hydroxypropylation of DNA. An alternative mechanism of activation involving sulfation of NDHPA cannot be excluded as a possible route leading to hydroxypropylation of DNA in this model.

### Acknowledgement

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# **METABOLISM**



## RECENT FINDINGS ON THE METABOLISM OF $\beta$ -HYDROXYALKYLNITROSAMINES

C.J. Michejda, S.R. Koepke, M.B. Kroeger-Koepke & W. Bosan

*Laboratory of Chemical and Physical Carcinogenesis,  
LBI-Basic Research Program, NCI-Frederick Cancer Research Facility,  
Frederick, MD 21701, USA*

$\beta$ -Hydroxynitrosamines appear to be refractory to  $\alpha$ -oxidation, the common pathway of metabolism of simple dialkylnitrosamines. Some years ago, we postulated that nitrosamines bearing a hydroxyl in the  $\beta$  position may be activated to alkylating agents by metabolic transformation to sulfate conjugates. Recent evidence has provided support for this hypothesis. A sulfate ester of *N*-nitroso(2-hydroxypropyl)(2-oxopropyl)amine (NHPOPA) has been found in the urine of hamsters treated with the nitrosamine. It has also been found that inhibition of sulfotransferases inhibited the development of DNA single-strand breaks in livers of rats treated with several  $\beta$ -hydroxy-nitrosamines. Alkylation of rat liver DNA *in vivo* by *N*-nitroso(2-hydroxyethyl)methylamine (NHEMA) favoured methylation over 2-hydroxyethylation by a factor of 10. The methylation reaction was inhibited by sulfotransferase inhibitors. Thus, sulfation appears to be an important pathway for activation of  $\beta$ -hydroxy-nitrosamines. There are, however, other pathways, such as the oxidation of the  $\beta$ -hydroxyl group to a carbonyl, which may also result in the formation of electrophilic species capable of modifying cellular macromolecules.

*N*-Nitrosodiethanolamine (NDELA) is very widespread in the human environment. It was found to be present in significant quantities in such disparate sources as metal-working fluids (Fan *et al.*, 1977a), tobacco products (Hoffmann *et al.*, 1984a) and cosmetics (Fan *et al.*, 1977b). NDELA was found to be a potent carcinogen in rats (Lijinsky *et al.*, 1980; Preussmann *et al.*, 1982) and must therefore be viewed as a potential human carcinogen. Other  $\beta$ -hydroxynitrosamines may be formed by metabolic transformations of dialkyl-nitrosamines. For example, NHEMA has been reported as a metabolic product of *N*-nitrosoethylmethylamine in rats (von Hofe *et al.*, 1986b). Some  $\beta$ -hydroxynitrosamines provide excellent animal models for human cancers. Thus, *N*-nitrosobis(2-hydroxypropyl)amine was found to induce a high yield of adenocarcinomas of the pancreas in hamsters (Pour *et al.*, 1974), a cancer similar to that commonly observed in humans. It appears, therefore, that  $\beta$ -hydroxynitrosamines constitute an important group of carcinogens and that some of them may contribute to human carcinogenesis.

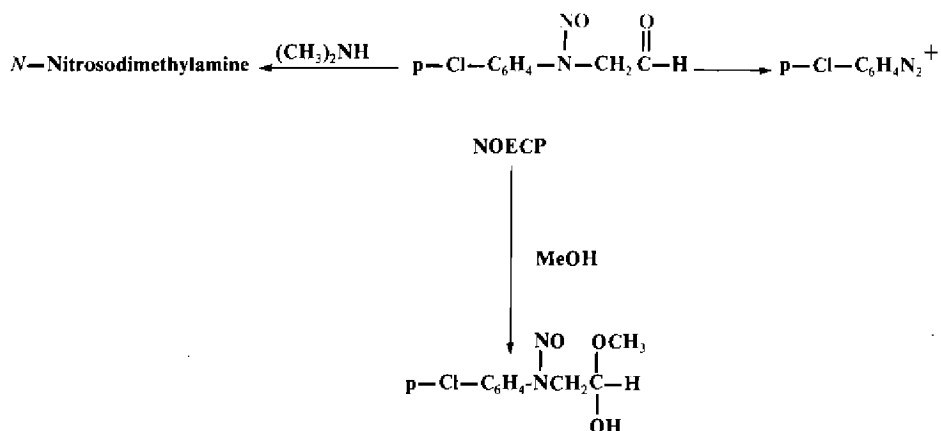
### Metabolic studies

Until recently, relatively little was known about the metabolism of these compounds. Unlike simple dialkylnitrosamines, the  $\beta$ -hydroxynitrosamines are not metabolized by microsomes. NDELA was found to be refractory to microsomal oxidation but was metabolized to an unidentified metabolite by isolated rat hepatocytes (Farrelly *et al.*, 1984). This experiment suggested that  $\alpha$ -hydroxylation, which appears to be mainly a cytochrome P450-catalysed microsomal oxidation, is not important in the bioactivation of NDELA. A



carcinogenic properties of 2-hydroxyethylnitrosamines. Recently, it was shown that NDELA, which is not mutagenic in the conventional *Salmonella typhimurium* mutagenesis assay, was transformed to a potent mutagen by in-vitro activation with alcohol dehydrogenase (Eisenbrand *et al.*, 1984b).

Scheme 2



The product formed during the alcohol dehydrogenase oxidation of NDELA is the corresponding aldehyde, *N*-nitroso(2-hydroxyethyl)ethanolamine, which cyclizes to its hemiacetal form, *N*-nitroso-2-hydroxymorpholine (NHMOR). This compound was shown to be a directly-acting mutagen (Hecht, 1984). Airoidi *et al.* (1983a) showed that NDELA was transformed to NHMOR and to its open form, as well as to the corresponding acid, *N*-nitroso(2-hydroxyethyl)carboxymethylamine by rat liver S9. The acid was also identified in the urine of rats treated with NDELA (Airoidi *et al.*, 1984).

### Sulfate conjugation

Recently, Sterzel and Eisenbrand (1986) provided some strong evidence that activation of  $\beta$ -hydroxynitrosamines to genotoxic metabolites involves conjugation with sulfate. These authors found that the inhibition of sulfotransferase by 2,6-dichloro-4-nitrophenol *in vivo* completely prevented DNA single-strand breaks induced by NDELA and NHMOR. In contrast, the strand breaks produced by *N*-nitrosodiethylamine were not affected by 2,6-dichloro-4-nitrophenol, and single-strand breaks induced by *N*-nitrosoethyl(2-hydroxyethyl)amine were partially inhibited by this compound. Partial inhibition of NHEMA was also observed (Sterzel & Eisenbrand, private communication).

Direct evidence for the formation of sulfate esters of  $\beta$ -hydroxynitrosamines was obtained by Kokkinakis *et al.* (1985). Rats and Syrian hamsters were treated with NHPOPA. The compound produces ductal adenocarcinoma of the pancreas in hamsters and has consequently been the subject of extensive investigations. The urinary metabolites of NHPOPA in the rat included unchanged NHPOPA, the reduction product, *N*-nitrosobis(2-hydroxypropyl)amine, and the glucuronides of both of these nitrosamines.

Hamster urine contained a smaller proportion of NHPOPA than rat urine, a somewhat larger amount of *N*-nitrosobis(2-hydroxypropyl)amine, and their respective glucuronides. However, it also contained a significant amount of NHPOPA sulfate ester, which was absent from rat urine. More recently, Kokkinakis *et al.* (1986) extended these studies to indicate that the excreted sulfate conjugate of NHPOPA is probably the *anti* isomer (i.e., nitroso oxygen pointing away from the sulfate group). Presumably the *syn*-isomer cyclizes rapidly to the reactive oxadiazolium ion, as proposed by Michejda *et al.* (1979). Moreover, they found that the rat liver cytosol was incapable of transforming NHPOPA to the sulfate conjugate, while hamster liver cytosol catalysed the sulfation reaction with an apparent  $V_{\max}$  of 0.2 nmol/min per mg protein. Those investigators concluded that it is likely that sulfation of  $\beta$ -hydroxynitrosamines can lead to the activation of these molecules to ultimate carcinogens.

### Studies on *N*-nitroso(2-hydroxyethyl)methylamine

NHEMA was found to be a liver carcinogen in rats (Koepke & Michejda, unpublished data). In contrast to most nitrosamines, this prototypical  $\beta$ -hydroxynitrosamine exhibited a significant sex difference in tumour distribution. Thus, the principal cause of death in female Fischer 344 rats was hepatocellular carcinoma (14/20 rats), whereas male rats had fewer liver tumours (6/20), but several males (4/20) exhibited nasal-cavity squamous-cell carcinomas which were absent in females. The chemical was administered twice weekly by gavage in corn oil (2.7 mg/dose) over the lifetime of the animals. The next highest homologue, *N*-nitroso(3-hydroxypropyl)methylamine, was found to be a much weaker carcinogen when administered at an equimolar dose (3 mg/dose), with the lung being the principal target for the chemical. Interestingly, NHEMA was more acutely toxic in male rats than in females. Thus, at a dose of 4 mg per application, 13/20 male rats died of cardiovascular failure within six weeks of start of treatment, while only 4/20 of the (smaller) females died early at the same dose.

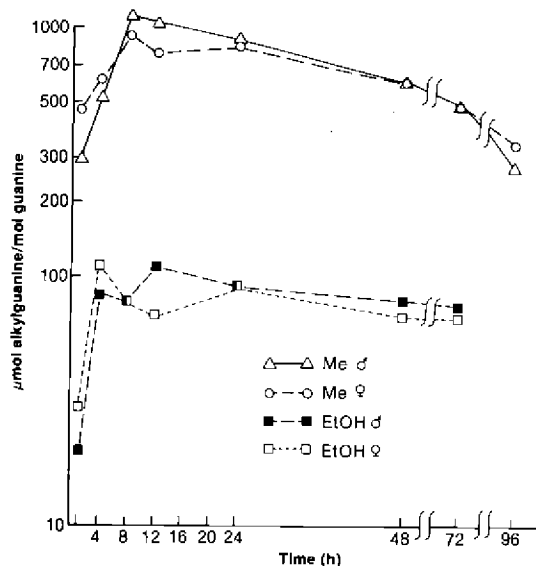
In an effort to learn more about the possible modes of activation of NHEMA and also to shed some light on the sexual dimorphism observed in its carcinogenicity, we studied the alkylation of DNA *in vivo*. The DNA from livers was isolated by a modified phenol extraction method and was relatively free from protein and RNA. Analysis of the DNA was carried out essentially according to the method of Herron and Shank (1979). Purified DNA was subjected to neutral (pH 7.0) thermal (100°C) hydrolysis for 35 min. This treatment effectively released guanine residues which had been alkylated at the *N7* position. The remaining DNA was then hydrolysed under mildly acidic conditions (0.1 M hydrochloric acid at 70°C for 45 min), which resulted in the release of all the other bases, including the guanines alkylated at the *O6* position. Both hydrolysates were analysed by high-performance liquid chromatography on a strong cation-exchange column. The effluent peaks were detected by fluorescence spectroscopy. Since only *N7* and *O6* alkylated guanines are strongly fluorescent, this method provides an excellent tool for the study of alkylation without recourse to radiolabelled substrates. An obvious disadvantage is that only alkylated guanines are detected; minor modified bases still require the use of radioactive reagents. Thus, the data presented below refer to four adducts, *N7*-methylguanine (7meGua), *N7*-(2-hydroxyethyl)guanine (7etOHGua), *O6*-methylguanine (*O6*-meGua), and *O6*-(2-hydroxyethyl)-guanine (*O6*-etOHGua).

The dose-response of the alkylation was determined using doses of 1, 10, 25, 50 and 100 mg/kg NHEMA applied by gavage in corn oil. The animals were sacrificed 4 h after

treatment. Analysis of the hydrolysates of liver DNA indicated that the alkylation was roughly linear with doses up to about 50 mg/kg. The data also indicated that after 4 h there is no great difference in the total alkylation between the two sexes. The most striking conclusion to be drawn from these data is that methylation predominates over hydroxyethylation by about a factor of 10. Moreover, the ratio of  $O^6$  to  $N^7$  methylation is about 0.1, a result similar to that observed with classical methylating agents such as *N*-nitrosodimethylamine and *N*-methyl-*N*-nitrosourea. The ratio  $O^6$ : $N^7$  for hydroxyethylation was found to be about 0.7.

We also studied the persistence of the alkylated lesions. Animals of each sex were treated with 25 mg/kg NHEMA in corn oil. The animals (five per point) were sacrificed at 0, 1, 4, 8, 12, 24, 48, 72 and 96 h after treatment. These data are displayed graphically in Figure 1 for persistence of the  $N^7$  adducts and in Figure 2 for the persistence of the  $O^6$  adducts. The alkylation at both sites by both groups increased up to about 12 h. At this point, the  $O^6$  lesions began to disappear rapidly and were essentially at background levels by 48 h. Remarkably, the disappearance of the  $O^6$ -etOHGua was just as fast as the disappearance of  $O^6$ -meGua (Fig. 2). This result is not consistent with the relative rate of repair of these lesions by  $O^6$ -alkylguanine-DNA alkyltransferase, where the relative rate ratio *in vitro* for  $O^6$ -meGua versus  $O^6$ -etOHGua was found to be 1:>50 (Pegg *et al.*, 1984). It must be remembered that the present experiment was carried out *in vivo* and measured persistence, not repair. The decrease of the lesions *in vivo* is a complex function of repair (dealkylation or possibly other repair mechanisms) and also of dilution of the alkylated bases by enhanced proliferation of liver cells in response to toxic injury, and possibly other factors. It is clear, however, that additional data will be needed to explain the rapid removal of the  $O^6$ -etOHGua lesions in our system. The  $N^7$  lesions (7-meGua and 7-etOHGua) reached a maximum in 8-12 h after treatment. The livers of males contained a little more of both lesions (e.g., for 7-meGua, the maximum amount for males was 1200  $\mu$ mol/mol guanine, while it was 900  $\mu$ mol/mol guanine for females). These maximum values declined slowly thereafter, but a significant amount remained, even at 96 h. Thus, the  $N^7$ -alkylguanines persisted long after the  $O^6$ -alkylguanines had disappeared.

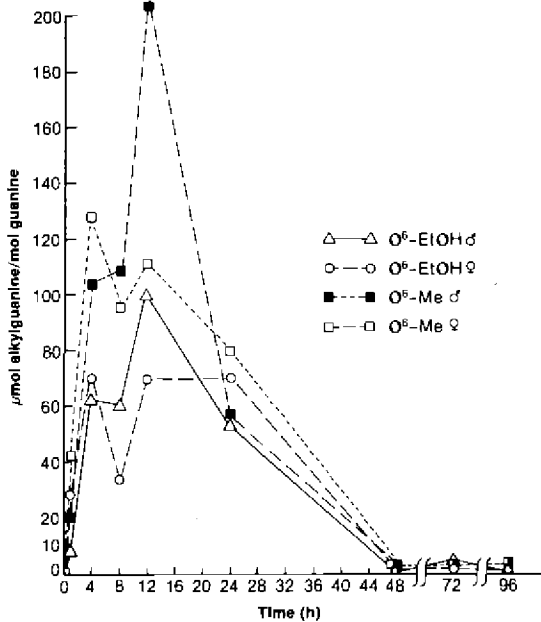
**Fig. 1. Persistence of  $N^7$ -alkylguanines in liver DNA of male and female Fischer 344 rats treated with 25 mg/kg NMHEA by gavage**



Treatment of calf thymus DNA with NHEMA-*O* tosylate (*p*-toluenesulfonate) in aqueous buffer at pH 7.4, followed by reisolation, purification and hydrolysis of the DNA as described above, indicated that this material methylated the guanine residues and that the  $O^6$ / $N^7$  ratio was about 0.1, similar to that obtained for NHEMA *in vivo*. On the basis of our earlier chemical studies (Koepke *et al.*, 1979), we know that the tosylate (a surrogate for the very unstable sulfate) cyclizes very rapidly to the oxadiazolium ion (Scheme 1). Thus, the methylation of genomic DNA by NHEMA *in vivo* is consistent with activation of this carcinogen by the

action of sulfotransferases. Consequently, we treated groups of rats intraperitoneally with 26  $\mu\text{mol/kg}$  2,6-dichloro-4-nitrophenol in propanediol 2 h before treatment by gavage

**Fig. 2. Persistence of  $O^6$ -alkylguanines in liver DNA of male and female Fischer 344 rats treated with 25 mg/kg NMHEA by gavage**



NDELA, this inhibition was incomplete for *N*-nitrosoethyl(2-hydroxyethyl)amine and NHEMA. They proposed that the genotoxic damage that could not be inhibited must be due to electrophiles produced by  $\alpha$ -hydroxylation. However, we found that NHEMA did not appear to be activated by  $\alpha$ -hydroxylation in hepatocytes. While we have not made the necessary in-vivo measurements of  $\alpha$ -hydroxylation in that compound, our previous experience suggests that hepatocytes are a reasonable model for the in-vivo system. Loeppky *et al.* (1984) suggested that oxidation of the 2-hydroxyethyl group on a nitrosamine to an aldehyde constitutes a potential activation pathway, since such aldehydes have the capacity to alkylate guanosine *in vitro* (Loeppky, private communication). This observation indicates that the  $\beta$ -hydroxynitrosamines may have more than one pathway of activation to electrophilic intermediates. The challenge now will be to determine which of these pathways is responsible for initiation of carcinogenesis.

#### Acknowledgements

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in corn oil with 25 mg/kg NHEMA. Controls were given NHEMA in corn oil, propanediol 2 h before NHEMA by gavage in corn oil, or 2,6-dichloro-4-nitrophenol in propanediol without the nitrosamine. The animals were sacrificed after 4 h. Preliminary data indicated that the sulfation inhibitor decreased the levels of alkylation of guanine substantially at both the *N7* and *O6* positions and that both methylation and 2-hydroxyethylation were affected. It appears that inhibition of sulfotransferases decreases the extent of genomic alkylation by NHEMA, in concordance with the results on DNA single-strand breaks observed by Sterzel and Eisenbrand (1986). Thus, sulfation of the  $\beta$ -hydroxyl group may indeed be an important activation pathway for  $\beta$ -hydroxynitrosamines.

Finally, Sterzel and Eisenbrand found that while 2,6-dichloro-4-nitrophenol was able completely to abolish single-strand breaks induced by

# IN-VIVO ACTIVATION OF *N*-NITROSODIETHANOLAMINE AND OTHER *N*-NITROSO-2-HYDROXYALKYLAMINES BY ALCOHOL DEHYDROGENASE AND SULFOTRANSFERASE

E. Denkel, W. Sterzel & G. Eisenbrand

*Department of Food Chemistry and Environmental Toxicology,  
University of Kaiserslautern, Kaiserslautern, Federal Republic of Germany*

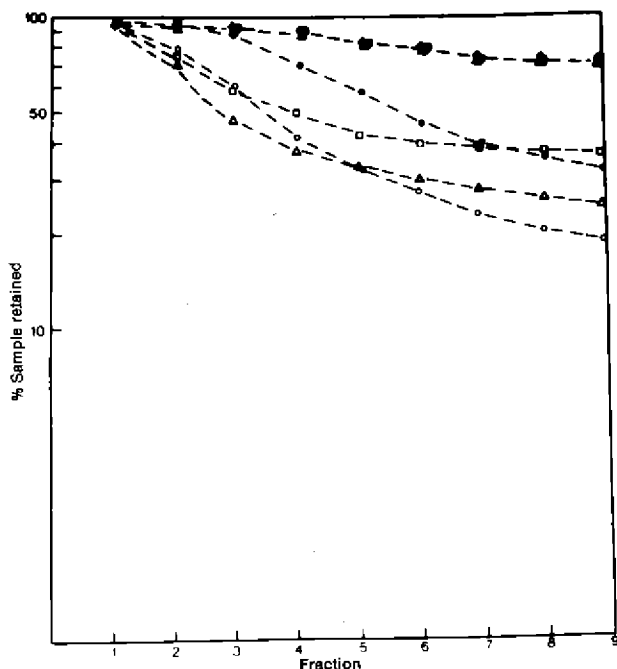
Alcohol dehydrogenase (ADH) activates *N*-nitrosodiethanolamine (NDELA) to a potent mutagen in the Ames mammalian microsome mutagenicity assay. *In vivo*, NDELA, its metabolite *N*-nitroso-2-hydroxymorpholine (NHMOR) and other 2-hydroxylated *N*-nitrosoalkylamines induce single-strand breaks in rat liver after a single oral application. After competitive inhibition of ADH by pretreatment with ethanol, induction of single-strand breaks by NDELA and *N*-nitroso(2-hydroxyethyl)ethylamine (NHEEA) was completely suppressed, whereas breaks induced by NHMOR were only partially reduced. Ethanol also influences cytochrome P450-dependent monooxygenases. To investigate whether the observed effect depends on inhibition of ADH and/or of monooxygenases, rats were pretreated with the ADH inhibitor 3-butylthiolane-1-oxide; a considerable reduction in the single-strand-break-inducing potential of NDELA was seen. Moreover, DNA damage induced by NDELA, NHMOR and other hydroxylated *N*-nitroso compounds is strongly reduced by pretreatment with the sulfotransferase inhibitor, 2,6-dichloro-4-nitrophenol (DCNP). DCNP pretreatment completely suppressed the induction of single-strand breaks by NDELA, whereas the number induced by NHEEA was only partially reduced. Our data suggest that ADH and sulfotransferase are enzymes responsible for the in-vivo activation of *N*-nitroso-2-hydroxyalkylamines.

The potent carcinogen NDELA has been shown to become strongly mutagenic to *Salmonella typhimurium* TA98 and TA100 when activated by alcohol dehydrogenase (NAD/ADH) (Eisenbrand *et al.*, 1984b). NHMOR has been identified by us as a product of ADH-mediated oxidation of NDELA. This result, together with the detection of *N*-nitroso(2-hydroxyethyl)glycine as a metabolite in the urine of rats treated with NDELA (Airoldi *et al.*, 1983a), indicates that metabolic  $\beta$ -oxidation of NDELA might be important for its in-vivo activation. Other  $\beta$ -hydroxylated nitrosamines are also substrates for ADH; therefore, activation by  $\beta$ -oxidation might be relevant for a broader spectrum of *N*-nitroso-2-hydroxyalkylamines.

As an alternative to  $\beta$ -oxidation, sulfate conjugation has been discussed as a possible pathway for activation of  $\beta$ -hydroxylated *N*-nitroso compounds (Michejda *et al.*, 1979; Sterzel & Eisenbrand, 1986). Our aim was to estimate the importance of these biotransformation steps in the genotoxic effects of NDELA and other *N*-nitroso-2-hydroxyalkylamines. Since the primary metabolites resulting from  $\beta$ -oxidation and sulfation are unstable, we tried to prove our hypothesis by studying the influence of inhibitors of ADH and sulfotransferase on the induction of single-strand breaks in DNA of rat liver, as determined by the alkaline elution technique (Kohn *et al.*, 1981; Sterzel *et al.*, 1985).

NDELA, its metabolite NHMOR and other  $\beta$ -hydroxylated *N*-nitrosamines, such as NHEEA, induce single-strand breaks in DNA of rat liver after a single oral administration (Denkel *et al.*, 1986; Sterzel & Eisenbrand, 1986). After pretreatment of rats with ethanol (4 g/kg bw as a 25% aqueous solution), the genotoxic potential of NDELA and NHEEA was completely inhibited; single-strand breaks induced by NHMOR were also markedly reduced, but not totally suppressed (Fig. 1). Ethanol alone had no detectable effect on the elution rate of rat liver DNA. These results substantiate the hypothesis that NHMOR represents an intermediate in the activation sequence of NDELA (Eisenbrand *et al.*, 1984b; Hecht, 1984).

**Fig. 1.** Elution profile of liver DNA of rats treated orally with NDELA ( $\square$ ), NHMOR ( $\circ$ ; 0.75 mmol/kg) and NHEEA ( $\Delta$ ; 0.039 mmol/kg);  $\diamond$ , solvent control;  $\blacksquare$ ,  $\bullet$ ,  $\blacktriangle$ , experiments with 4 g/kg ethanol

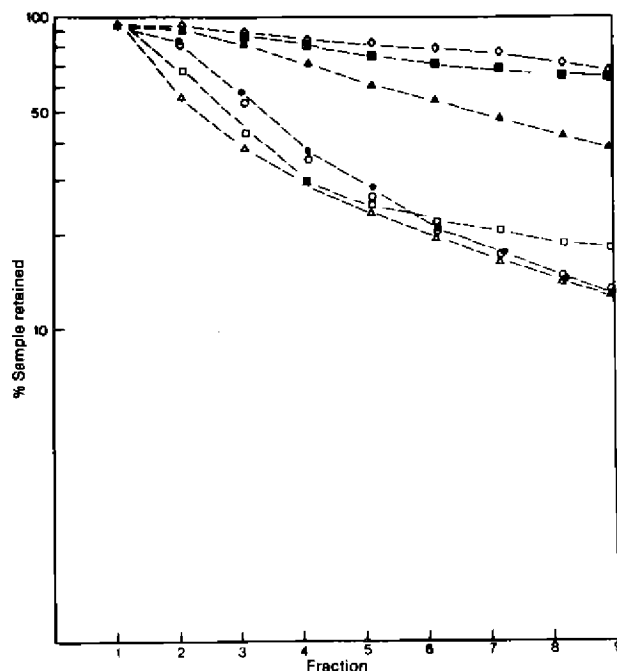


pretreatment. This result suggests that NHEEA is indeed metabolized by ADH as well as by monooxygenases. The genotoxic potential of NHMOR was not suppressed at all by 3-butylthiolane-1-oxide, and there was only partial inhibition of the genotoxic effectiveness of NHMOR after ethanol pretreatment. The genotoxic activity of this compound must therefore be due to further activation by monooxygenases, and possibly also by other enzymatic systems (e.g., sulfotransferases).

Ethanol also influences other enzymatic systems, especially cytochrome P450-dependent monooxygenases (Reinke *et al.*, 1983). It was therefore investigated whether the suppression of genotoxic effects was due to inhibition of ADH or of monooxygenases. A case in point is NHEEA: this unsymmetrically substituted *N*-nitrosamine might be activated by ADH at the  $\beta$ -hydroxy position, but it might equally well be  $\alpha$ -C-hydroxylated by monooxygenases at the ethyl group. Since both enzymatic oxidations are blocked by ethanol, we attempted a specific inhibition of ADH by using 3-butylthiolane-1-oxide. Pretreatment of rats with this uncompetitive inhibitor resulted in almost complete disappearance of the single-strand-break-inducing potential of NDELA (Fig. 2). The DNA-damaging properties of NHEEA, however, were only partially reduced, in contrast to the total inhibition seen after ethanol



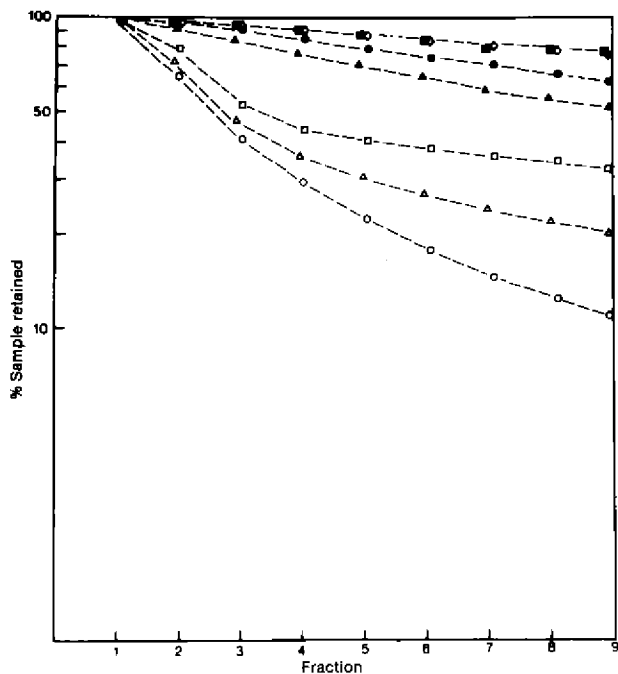
Fig. 2. Elution profile of liver DNA of rats treated orally with solvent ( $\diamond$ ), NDELA ( $\square$ ), NHMOR ( $\circ$ ; 0.75 mmol/kg) and NHEEA ( $\triangle$ ; 0.039 mmol/kg);  $\blacksquare$ ,  $\bullet$ ,  $\blacktriangle$ , experiments with 1 mmol/kg 3-butylthiolane-1-oxide injected intraperitoneally 0.5 h before application of the *N*-nitroso compound



Pretreatment of rats with the sulfotransferase-inhibitor 2,6-dichloro-4-nitrophenol (DCNP; Sterzel *et al.*, 1985) resulted in complete suppression of the DNA-damaging properties of NDELA (Fig. 3), and the single-strand break potential of NHMOR was suppressed to a small residual level. This result can be reconciled with the concept of a two-step metabolic activation: NDELA is first  $\beta$ -oxidized by ADH to the corresponding aldehyde, which rapidly cyclizes to its hemiacetal NMHOR. The latter is a substrate for sulfotransferases. Sulfation of NHMOR results in the generation of a highly reactive electrophilic species, which we consider to be the ultimate carcinogen.

Pretreatment with DCNP results in partial suppression of the DNA-damaging effectiveness of NHEEA (Fig. 3). These results clearly show that direct activation of NHEEA by sulfotransferase is responsible in part for its genotoxic potential. The same appears to be true for activation by ADH and cytochrome P450-dependent monooxygenases, which also account in part for the genotoxic effects of NHEEA. NHEEA therefore appears to be a substrate for ADH, for sulfotransferase and for monooxygenases.

**Fig. 3.** Elution profile of liver DNA of rats treated orally with solvent ( $\diamond$ ), NDELA ( $\square$ ), NHMOR ( $\circ$ ; 0.75 mmol/kg) and NHEEA ( $\Delta$ ; 0.039 mmol/kg);  $\blacksquare$ ,  $\bullet$ ,  $\blacktriangle$ , experiments with 26  $\mu$ mol/kg DCNP injected 2 h before administration of the corresponding nitrosamine



## METABOLISM AND CELLULAR INTERACTIONS OF *N*-NITROSODIETHANOLAMINE

J.G. Farrelly, B.J. Thomas & W. Lijinsky

*NCI-Frederick Cancer Research Facility,  
LBI-Basic Research Program, Frederick, MD 21701, USA*

*N*-Nitrosodiethanolamine (NDELA) labelled with  $^{14}\text{C}$  at the  $\alpha$  carbon was administered by gavage to adult male Fischer 344 rats at various doses ranging from 0.6 to 100 mg per rat. The proportion of the dose excreted as  $^{14}\text{CO}_2$  was small, ranging from 0.27% at the lowest dose to 0.83% at the highest in 24 h. At all doses, approximately 95% of the dose of radioactivity (most of which was NDELA) appeared in the urine within 24 h, but the proportion of metabolites increased from 7% to 14% from the lowest to the highest dose. The specific activity of the nucleic acids isolated from the liver of rats given 100 mg and 100  $\mu\text{Ci}$  of NDELA was very low and was the same at 6 h and 24 h after treatment (70 dpm/mg DNA, 92-95 dpm/mg RNA). *N*7-(2-Hydroxyethyl)guanine and *O*<sup>6</sup>-(2-hydroxyethyl)guanine were tentatively identified in the hydrolysates of the nucleic acids, comprising 10% and 4%, respectively, of the DNA radioactivity; there was no difference between the amounts found 6 h and 24 h after NDELA treatment. In addition to NDELA, four components were separated from rat urine, and two were identified. One is the glucuronide of NDELA, the other is *N*-nitroso-*N*-(2-hydroxyethyl)carboxymethylamine. Neither nitroso-2-hydroxymorpholine nor a sulfate of NDELA was detected.

The important environmental carcinogen NDELA is a broadly-acting compound that induces tumours of the liver, nasal cavity, kidney and oesophagus in rats, and tumours of the nasal cavity, trachea and lung in Syrian hamsters. The inactivity of NDELA in short-term assays for carcinogenic potential and its low toxicity to animals suggest that its mechanism of action might be unusual. We have investigated the metabolism and interactions of NDELA with liver macromolecules in rats.

Using  $^{14}\text{C}$ -NDELA (labelled on the  $\alpha$  carbon), a series of doses was administered in water by gavage to male Fischer 344 rats, and the  $^{14}\text{CO}_2$  expired over 24 h was collected and counted (Table 1). Each of the doses corresponded to the weight of NDELA administered each week to one group of rats in an extensive dose-response study we conducted, in which all dose levels but the lowest gave rise to liver tumours in male rats (Lijinsky & Reuber, 1984; Lijinsky & Kovatch, 1985). The urine collected from the animals over 24 h was assayed for radioactivity, and the labelled components were separated by high-performance liquid chromatography (HPLC). The urine from the highest-dose treatments (100 mg NDELA containing 100  $\mu\text{Ci}$ ) was used for isolation and identification of the principal metabolites. From the same group of six rats given 100 mg NDELA, two were sacrificed at 6 h, and the remainder at 24 h, and the livers were homogenized prior to isolation of nucleic acids and soluble protein, following our usual procedure (Lijinsky & Ross, 1969). To determine the

nature of the interaction of the metabolites of NDELA with liver DNA and RNA, 20-30 mg of each nucleic acid (which had very low specific activity) were hydrolysed (DNA in 0.1 N hydrochloric acid at 75°C for 1 h; RNA in 1 N hydrochloric acid at 100°C for 1 h). Aliquots of the hydrolysates containing at least 500 dpm were chromatographed by HPLC on a Whatman SCX ion-exchange column using 0.2 M phosphate buffer, pH 3.5, at 0.8 ml/min. Samples were collected every 15 sec and counted. Several radioactive peaks were present in the nucleic acid hydrolysates, two of which coincided in retention time with standards of *N*7-(2-hydroxyethyl)guanine (6 min) and *O*<sup>6</sup>-(2-hydroxyethyl)guanine (7.5 min). In the DNA hydrolysates, these two radioactive peaks contained, respectively, 10% and 4% of the radioactivity in the DNA. Table 2 shows these results based on 1 mg nucleic acid; the specific activity of the soluble (methanol-precipitated) protein is given for comparison.

**Table 1. Excretion of <sup>14</sup>C over 24 h by adult male rats given <sup>14</sup>C-NDELA<sup>a</sup>**

Dose of NDELA (mg per rat)	Percentage as <sup>14</sup> CO <sub>2</sub>	Percentage of <sup>14</sup> C in urine	
		Total	As meta- bolites
100 mg (100 μCi <sup>14</sup> C)	0.83	95	13.9
50 mg (100 μCi <sup>14</sup> C)	0.40	96	12.9
8 mg (100 μCi <sup>14</sup> C)	0.49	96	12.5
3 mg (7 μCi <sup>14</sup> C)	0.12	95	9.2
1.3 mg (7 μCi <sup>14</sup> C)	0.28	94	9.7
0.6 mg (7 μCi <sup>14</sup> C)	0.27	95	7.4

<sup>a</sup>Each rat was given 0.2 ml of a solution of <sup>14</sup>C-NDELA in water by gavage. Animals were placed in a closed metabolism cage to which air was supplied slowly; the exit gas was bubbled into 10 ml of ethanolamine:methanol (1:1), which was changed every hour. The urine collected over 24 h was counted, and an aliquot was chromatographed on HPLC, to separate the large component, which was unchanged NDELA, from the metabolites. All fractions were assayed for <sup>14</sup>C activity by liquid scintillation counting.

somal oxidases in rat liver (Farrelly *et al.*, 1984), but it is metabolized by other enzymes in rat liver. However, it is not yet known which of these routes of metabolism is involved in the carcinogenesis of NDELA.

Table 2 shows the specific activity of the nucleic acids and proteins isolated from rat liver 6 and 24 h after administration of 100 mg NDELA containing 100 μCi <sup>14</sup>C. Labelling of the soluble protein was higher than that of the nucleic acids, contrary to what is observed with most *N*-nitroso compounds.

The specific activity of the liver RNA was somewhat greater than that of DNA. This low specific activity of the macromolecules is consistent with the low metabolism of NDELA in rat liver. The liver of one rat was processed to isolate nuclei and mitochondria: labelling of the nuclei corresponded to 0.0025 μCi/10 g liver and that of the mitochondria, 0.01 μCi/10 g liver. The specific activities of the nucleic acids did not change between 6 and 24 h after administration of NDELA, showing that there was little turnover or removal of the label.

As shown in Table 1, at all doses from 0.6 mg to 100 mg, approximately 95% of the dose was excreted in the urine within 24 h; most of the radioactivity in the urine was unchanged NDELA, but approximately 10% was in the form of metabolites. The proportion of the dose excreted as <sup>14</sup>CO<sub>2</sub> was always less than 1%, but was greater at the high doses than at the low doses. We conclude that the amount of NDELA converted to metabolites that enter the carbon pool of the rat is very small, as compared, for example with *N*-nitrosodimethylamine or *N*-nitrosodiethylamine, for which the corresponding amount is 70% or more. It has been shown that NDELA is not metabolized by micro-

**Table 2. Interaction of NDELA with rat liver macromolecules (each of six male rats received 100 mg, 100  $\mu$ Ci  $^{14}$ C-NDELA by gavage)**

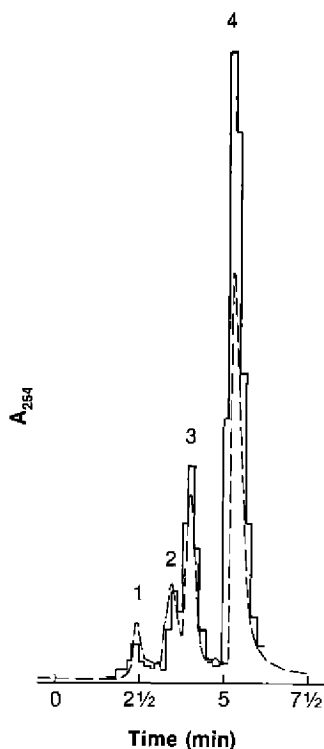
Radioactivity (dpm)	6 hours (2 rats)	24 hours (4 rats)
<i>DNA</i> (per mg)	71	71
as <i>N</i> 7-(2-hydroxyethyl)guanine (per mg DNA)	6	6.5
as <i>O</i> 6-(2-hydroxyethyl)guanine (per mg DNA)	3	2.5
<i>RNA</i> (per mg)	95	92
as <i>N</i> 7-(2-hydroxyethyl)guanine (per mg RNA)	22	25
<i>Soluble protein</i> (per mg)	500	430

with *O*6-(2-hydroxyethyl)guanine, and comprised approximately 4% of the DNA radioactivity. In these limited studies, there was no indication that the alkylated bases were removed between 6 and 24 h after NDELA treatment, although the possibility of some DNA repair cannot be excluded. The biological significance of the extremely small alkylation of DNA produced in rat liver by NDELA (approximately one alkylated base in  $10^6$ - $10^7$ ) cannot be assessed. However, if it is significant, it calls into question assumptions made about the relation of the enormously greater extents of alkylation that have been observed with such nitrosamines as *N*-nitrosodimethylamine and *N*-nitrosodiethylamine, which are no more than two orders of magnitude more potent than NDELA as carcinogens.

We have attempted the isolation and identification of metabolites of NDELA that appear within 24 h in the urine of rats given 100  $\mu$ Ci. Three ml of urine from rats treated with 100 mg NDELA were cleared by centrifugation and placed on a  $2.5 \times 90$  cm Sephadex G-10 column and eluted with water at 1 ml/min. Six-ml fractions were collected, and an aliquot from each was counted in 5 ml of scintillation fluid. The metabolites that eluted between fractions 36-46 were pooled and concentrated under a stream of nitrogen in the cold. After passage of the concentrated pool through a Waters Sep-Pak  $C_{18}$  cartridge, aliquots were chromatographed on a  $4.6 \times 250$  mm Whatman Partisil 10 SAX column eluted with 0.05 M sodium phosphate buffer pH 3.5 at 1.5 ml/min. Fractions were collected, and an aliquot of each was counted in 5 ml of scintillation fluid. The elution pattern is shown in Figure 1. In an effort to identify the peaks (marked 1 to 4 in the figure), 12 separate aliquots were chromatographed and each peak pooled. Peak 1 co-chromatographed with NDELA and is probably a small amount of contaminating starting material. Peak 2, when treated with  $\beta$ -glucuronidase and subjected to HPLC on the SAX column, shifted its elution position and co-eluted with peak 1. The kinetics of the  $\beta$ -glucuronidase reaction did not change in the presence of two concentrations (25 and 250  $\mu$ M) of 2,6-dichlorophenol, a sulfatase inhibitor. Thus, it appears that peak 2 is a glucuronide of NDELA. Peak 3 has not yet been identified.

Analysis of the hydrolysates of the nucleic acids also differed little in animals killed at 6 h and those at 24 h. Most of the radioactivity in the chromatograms has not yet been identified with a known product; however, a peak constituting approximately 10% of the total radioactivity, coinciding in retention time with 7-(2-hydroxyethyl)guanine, was present in the hydrolysates of the DNA and RNA samples. This peak represented approximately 10% of the DNA radioactivity and 25% of the RNA activity. This alkylated purine was previously tentatively identified as a metabolite of *N*-nitrosomorpholine in rats (Stewart *et al.*, 1974). A smaller peak in the DNA hydrolysates coincided in retention time

**Fig. 1. HPLC pattern of NDELA metabolites on a Whatman Partisil 10 SAX column eluted with 0.05 M sodium phosphate buffer pH 3.5**



The dashed lines indicate the absorbance at 254 nm, while the solid lines indicate the relative radioactivity of each sample. The numbers designate the radioactive peaks and are described in the text.

#### Acknowledgements

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Peak 4 was isolated in sufficient quantity to be analysed by proton nuclear magnetic resonance using a Nicolet NT-300 spectrometer with deuterated acetonitrile as solvent containing 0.5% tetramethylsilane; it exhibits a spectrum consistent with that of *N*-nitroso-(2-hydroxyethyl)(carboxymethyl)amine, which has previously been identified as a metabolite of NDELA (Airoldi *et al.*, 1983a). None of the metabolites co-chromatographed with *N*-nitroso-2-hydroxymorpholine, which has been reported to be a  $\beta$ -oxidation product of NDELA (Hecht, 1984).

## **$\beta$ -OXIDATION OF N-NITROSODIETHANOLAMINE IN DIFFERENT ANIMAL SPECIES *IN VITRO* AND *IN VIVO***

**M. Bonfanti, C. Magagnotti, R. Fanelli & L. Airoidi**

*Laboratory of Environmental Pharmacology and Toxicology,  
Istituto di Ricerche Farmacologiche 'Mario Negri',  
Via Eritrea 62, 20157 Milan, Italy*

The metabolism of *N*-nitrosodiethanolamine (NDELA) was studied to assess whether the formation of the  $\beta$ -oxidized metabolites, *N*-nitroso(2-hydroxyethyl)(formylmethyl)amine (NHEFMA) and *N*-nitroso(2-hydroxyethyl)(carboxymethyl)amine (NHECMA), is involved in tumour induction in various animal species with different susceptibilities to NDELA carcinogenicity. *In-vitro* and *in-vivo* studies showed that all the animal species considered metabolize NDELA through the  $\beta$ -oxidation pathway, suggesting that there is no correlation between carcinogenesis by NDELA and its  $\beta$ -oxidation.

Oxidation of NDELA at the carbon-2 position, leading to the formation of the nitrosamino aldehyde NHEFMA and the nitrosamino acid NHECMA has been shown to occur *in vitro* and *in vivo* (Airoidi *et al.*, 1983a, 1984). It was considered that this biotransformation might represent an alternative metabolic pathway to  $\alpha$ -hydroxylation in the activation of this compound to a carcinogen or mutagen. The metabolism of NDELA was therefore investigated in an effort to clarify whether the formation of  $\beta$ -oxidized metabolites is involved in tumour induction in various animal species with different susceptibilities to NDELA carcinogenicity.

Hepatic S9 preparations (1 ml) from untreated rats, mice, hamsters and rabbits were incubated with 0.19 mM NDELA and 0.6 M  $\text{NAD}^+$  for 15 min at 37°C. Rats, mice and hamsters were housed individually in metabolic cages and given a single dose of 5 mg/kg NDELA intraperitoneally. Urine samples were collected on ammonium sulfamate 24, 48, 72 and 96 h after dosing. NDELA, NHEFMA and NHECMA were extracted and analysed quantitatively as their trimethylsilyl derivatives by gas chromatography-thermal energy analysis.

The amounts of NHEFMA and NHECMA formed *in vitro* after incubation of NDELA with hepatic S9 mix from different animal species and  $\text{NAD}^+$  are reported in Table 1. All the animal species tested metabolized NDELA through the  $\beta$ -oxidation pathway, with no apparent relation to their susceptibility to the compound's carcinogenic activity. Except for S9 from rabbits, which produced only about half the aldehyde formed by S9 from the other species, the total amount of NHEFMA formed did not differ significantly in the species tested. NHECMA formation, however, was greater in hamsters and rabbits than in rats and mice.

**Table 1. In-vitro metabolism of NDELA by hepatic S9 fractions from different animal species<sup>a</sup>**

Animal species	Unchanged NDELA		NHEFMA formed		NHECMR formed	
	nmol	%	nmol	%	nmol	%
Rat	123±3.2	65.9±1.7	0.18±0.01	0.09±0.005	1.48±0.12	0.79±0.06
Mouse	138±1.8	74.2±1.0	0.15±0.01	0.08±0.006	0.74±0.07	0.39±0.03
B6C3F1						
CD-1	128±4.8	68.4±2.6	0.16±0.01	0.09±0.007	0.83±0.002	0.44±0.0003
Hamster	111±7.9	58.9±4.1 <sup>b</sup>	0.16±0.02	0.09±0.013	5.1±0.5	2.7±0.27 <sup>c</sup>
Rabbit	97±6.3	51.7±3.4 <sup>d</sup>	0.07±0.005	0.04±0.002 <sup>e</sup>	5.2±0.3	2.8±0.17 <sup>f</sup>

<sup>a</sup>Values are means ± SE of at least four animals. Samples were incubated at 37°C for 15 min with 25 µg (187 nmol) NDELA and 0.6 mmol NAD<sup>+</sup>. Protein concentrations (mg/ml) were as follows: rat, 34; B6C3F1 mouse, 32; CD-1 mouse, 35; hamster, 29; rabbit, 21.

<sup>b</sup>*p* < 0.05 vs B6C3F1

<sup>c</sup>*p* < 0.01 vs rat, B6C3F1, CD-1

<sup>d</sup>*p* < 0.01 vs B6C3F1

<sup>e</sup>*p* < 0.05 vs rat, CD-1, hamster

<sup>f</sup>*p* < 0.01 vs rat, B6C3F1, CD-1 (Dunnett's test)

Figure 1 shows the overall elimination of unchanged NDELA and NHECMA; the 0-96-h excretion of NDELA was significantly higher in rats (44%) than in hamsters (34%) and mice (34% and 26%, respectively, for B6C3F1 and CD-1 mice). Conversely, rats eliminated the smallest amount of NHECMA (8%), followed by CD-1 mice (12%), B6C3F1 mice (14%) and hamsters (15%). The excretion of NDELA plus NHECMA over 96 h varied from 38% in CD-1 mice to 52% in rats.

The differences between our in-vitro and in-vivo data might be due to different hepatic and extrahepatic activities of alcohol/aldehyde dehydrogenase.

Our results suggest there is no correlation between NDELA carcinogenesis and its β-oxidation. In fact, B6C3F1 mice, which are reportedly (Lijinsky *et al.*, 1980) not susceptible to the carcinogenic activity of this nitroso compound, metabolize it through the β-oxidation pathway just as do susceptible species such as rats and hamsters. Hence, NDELA carcinogenicity must rely on its α-hydroxylation or on the trans-nitrosation capacity of NHEFMA or on an unknown metabolic pathway; all of these processes would account for the unrecovered part of the NDELA dose.

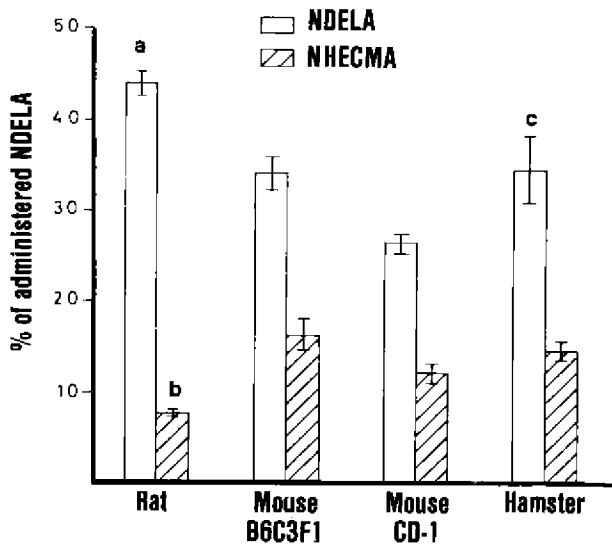
The results of the present study might indicate a detoxifying role of NHECMA formation.

#### Acknowledgements

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Fig. 1. 0-96-h urinary excretion of NDELA and NHECMA by different animal species after an intraperitoneal dose of 5 mg/kg



Columns represent means  $\pm$  SE of at least four animals.

a,  $p < 0.01$  vs CD-1, hamster;  $p < 0.05$  vs B6C3F1 mice; b,  $p < 0.01$  vs B6C3F1, CD-1, hamster; c,  $p < 0.05$  vs CD-1 (Dunnnett's test)

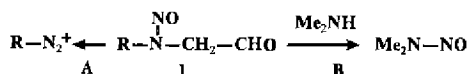
## $\alpha$ -NITROSAMINOALDEHYDES: HIGHLY REACTIVE METABOLITES

R.N. Loeppky<sup>1</sup>, W. Tomasik<sup>1</sup>, G. Eisenbrand<sup>2</sup> & E. Denkel<sup>2</sup>

<sup>1</sup>Department of Chemistry, University of Missouri, Columbia, MO 65211, USA;  
and <sup>2</sup>Department of Food Chemistry and Environmental Toxicology,  
Kaiserslautern University,  
6750 Kaiserslautern, Federal Republic of Germany

$\alpha$ -Nitrosamino aldehydes are highly reactive compounds which are directly-acting mutagens and are capable of facile transnitrosation to secondary and primary amines. The latter lead reactions to deamination. *N*-Nitrosobutyl(2-oxoethyl)amine (NBOEA) undergoes spontaneous decomposition in buffer at pH >7 (25°C) to give glyoxal and products implicating the formation of the butyl diazonium ion. NBOEA reacts with guanosine to produce xanthosine (by deamination), 7-butylguanosine and the 1,*N*<sup>2</sup> glyoxal adduct, among other products. All  $\beta$ -nitrosaminoethanols investigated undergo liver alcohol dehydrogenase-catalysed oxidation to their corresponding aldehydes. Several of these aldehydes have been shown to be directly-acting mutagens. These data provide strong evidence for an alternative carcinogenic bioactivation route for nitrosamines which does not involve  $\alpha$ -oxidation.

Loeppky *et al.* (1984) advanced the hypothesis that  $\alpha$ -nitrosaminoaldehydes could be directly-acting carcinogens produced by the enzymic oxidation of environmentally prevalent alkyl nitrosaminoethanols and that this could provide a new, alternative mode of nitrosamine bioactivation that does not involve  $\alpha$ -hydroxylation. The instability of NBOEA, first reported by Suzuki and Okada (1979), and our finding that an aryl- $\alpha$ -nitrosaminoaldehyde both decomposed to a diazonium ion and transnitrosated dimethylamine (Eq. 1) led to our suggestion that  $\alpha$ -nitrosaminoaldehydes could produce mutations and cell alteration by deamination of purine or pyrimidine bases or other amino groups and by alkylation of nucleic acids. In this paper we report the results of chemical and biochemical experiments devised to test this hypothesis and show that a single  $\alpha$ -nitrosaminoaldehyde can lead to three different types of nucleoside chemical alteration: deamination, alkylation and glyoxal adduct formation.



*N*-Nitrosodiethanolamine (NDELA) is probably the most common environmental nitrosamine and is known to be a potent carcinogen. Our analysis of metal-working fluids taken recently from a plant site in Minnesota (Garry *et al.*, unpublished data) revealed the presence of five nitrosamines, including the  $\beta$ -hydroxynitrosamines NDELA (0.4%) and *N*-nitrosomethylethanolamine (10 ppm), which may be involved in a raised cancer incidence at this plant site (Garry *et al.*, unpublished data). Alkyl nitrosaminoethanols have also been

implicated as important metabolites of larger nitrosamines. Suzuki and Okada (1985) have shown that *N*-nitrosobutylethanolamine (NBELA), a rat hepatocarcinogen, is produced in significant quantities from the metabolism of either of the rat hepatocarcinogens, *N*-nitrosobutyl-3-oxo- or -hydroxybutanamine. Those authors believe that NBELA is a metabolic intermediate in the production of 2-*N*-nitroso-*N*-butylaminoethanoic acid, the principal urinary metabolite of *N*-nitrosobutyl-3-oxo- or -hydroxybutanamine. The biochemical oxidation of alkylnitrosaminoethanols of environmental or metabolic origin could lead to highly reactive  $\alpha$ -nitrosaminoaldehydes.

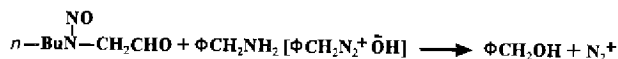
While research on the chemistry and biochemistry of  $\alpha$ -nitrosaminoaldehydes was underway in Missouri, Hecht (1984) and Eisenbrand *et al.* (1984b) showed independently that *N*-nitroso(2-hydroxy)morpholine (NHMOR) is a potent directly- and indirectly-acting mutagen (see references in Chung and Hecht, 1985, for a complete summary). NHMOR is a metabolite of both *N*-nitrosomorpholine and NDELA. Eisenbrand *et al.* (1984b) demonstrated that coincubation of alcohol dehydrogenase and NDELA resulted in the production of a potent mutagen. In addition to demonstrating its metabolic intermediacy, Airoidi *et al.* (1984) showed that NHMOR is capable of transnitrosating dibutylamine in aqueous solution.

Seeking support for our hypothesis, we first focused our attention on determining whether the transnitrosation reaction of the aryl  $\alpha$ -nitrosaminoaldehyde was also a property of its alkyl structural analogues. While the bulk of this work is being published elsewhere, the results are summarized here. Four  $\alpha$ -nitrosaminoaldehydes ( $R-NNO-CH_2CHO$ ,  $R = CH_2CH_2OH$ , Me, *n*-Bu, *t*-Bu) have been synthesized by the hydrolysis of their corresponding methyl acetals. They were purified by silica-gel chromatography followed by bulb-to-bulb distillation at low pressures and temperatures. Each of these compounds was found to react with dimethylamine to produce *N*-nitrosodimethylamine, in addition to other products (Eq. 1). The transnitrosation was most efficient when conducted in aprotic organic solvents such as benzene but also occurred in methanol and in water. Transnitrosation to morpholine, pyrrolidine and *N*-methylaniline was also observed.

The transnitrosation reaction of  $\alpha$ -nitrosaminoaldehydes can be followed by high-resolution Fourier transform nuclear magnetic resonance. In addition to other products, the aldimine CH signals of  $R-N=CH-CH=N-R$  can be seen to increase in a proportional manner to the transnitrosation product, *N*-nitrosodimethylamine. The formation of the imines of glyoxal has been corroborated independently by synthesis and gas chromatography-mass spectroscopy. In aqueous solution, glyoxal rather than its imines are observed. The transnitrosation reaction to dimethylamine or morpholine has been examined as a function of pH and occurs most rapidly between pH 8 and pH 9. The reaction is much slower in aqueous solution than in aprotic solvents. We presume that this is due to their predominant existence as 1,1-diols (hydrates), as is shown by nuclear magnetic resonance examination of  $D_2O$  solutions of the aldehydes (see below).

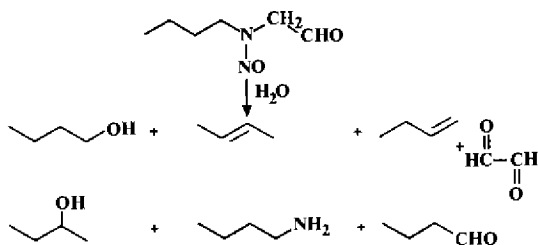
While the highly unusual and facile transnitrosation reaction of  $\alpha$ -nitrosaminoaldehydes to secondary amines is of considerable chemical interest, nitroso transfer to a primary amine is of greater biological significance because it will result in the formation of an unstable diazonium ion. Decomposition of the resulting diazonium ion will result in deamination and may involve the production of an alkylating agent. This possibility has been subjected to a cursory test. Reaction of NBOEA with benzylamine in benzene results in the production of the deamination product, benzyl alcohol, as well as small amounts of benzaldehyde (Eq. 2). When dimethylamine is present in the reaction mixture, competition between primary amine deamination and secondary amine transnitrosation is observed, and

the ratio of benzaldehyde to benzyl alcohol increases. Similar results have been obtained with other  $\alpha$ -nitrosaminoaldehydes and primary amines.



An understanding of the metabolic pathways for the carcinogenic activation of NDELA is important because of its widespread occurrence. From a chemical point of view, however, this goal is made more difficult by the fact that its oxidation product, NHMOR, is likely to be involved in an equilibrium with the open-chain aldehyde-alcohol. In order to understand the basic chemistry of the reactive  $\alpha$ -nitrosaminoaldehyde moiety, we first concentrated on the chemical properties of NBOEA, which has a straight alkyl chain bound to the nitrosamine nitrogen. When this  $\alpha$ -nitrosaminoaldehyde is permitted to decompose spontaneously in aqueous buffer (25°C), its half-life diminishes as the pH is increased from 7-9, while it appears to be reasonably stable at pH 5. The products of the decomposition of NBOEA are shown in Scheme 1. Because of the varied properties of the decomposition products, different analytical procedures have been used to detect different groups of products. Head-space analysis of the aqueous solution was utilized for gas chromatography-mass spectrometric detection of 1-butanol, 2-butanol, 1-butene and 2-butene. These products have their origin in the butyl carbocation from the butyl diazonium ion.

Scheme 1



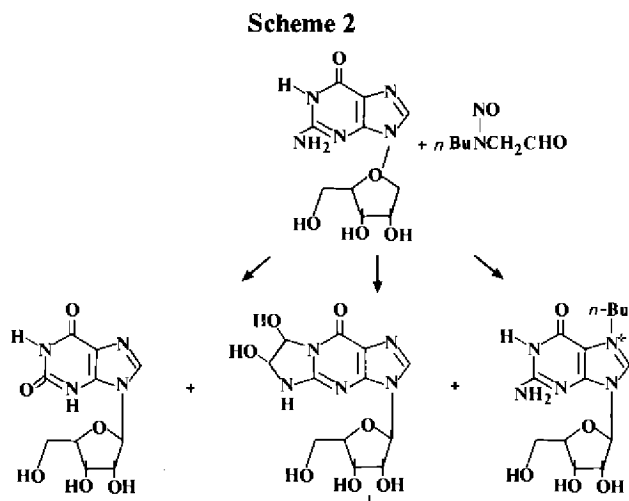
Semicarbazone and 2,4-dinitrophenylhydrazone derivatization of the aldehydes and ketones followed by high-performance liquid chromatography (HPLC) revealed the presence of butanal and glyoxal. It is not yet known whether glycolaldehyde is present in the mixture. Glycolaldehyde can give the same hydrazone (osazone) as glyoxal. Sensitive methods for determining the former without this

interference are being developed. Use of the Sanger reagent permitted the detection of butylamine; addition of dimethylamine to the reaction mixture resulted in the formation of *N*-nitrosodimethylamine, with greatest yield (9%) at pH 9. (Yields as high as 65% can be achieved in benzene at 25°C.) Addition of dimethylamine did not quench the formation of any of the other products, although yields of those originating from the *n*-butyl diazonium ion decreased qualitatively.

Further research has shown that  $\alpha$ -nitrosaminoaldehydes with various alkyl and hydroxyalkyl groups attached to the nitrogen all exhibit similar chemical properties. The nature of these data strongly suggests at least three different modes by which  $\alpha$ -nitrosaminoaldehydes could interact with appropriate DNA or RNA bases: (1) deamination of amino groups in guanosine, adenosine, cytidine or similar nucleoside residues; (2) alkylation of bases by the *N*-bound group opposite the  $\alpha$ -nitrosaminoaldehydes moiety; and (3) condensation of bases such as guanosine with glyoxal or its derivatives to give cyclic adducts.

In order to test this hypothesis, we recently examined the reaction of guanosine (0.005 M) with NBOEA (0.01 M) in phosphate buffer (0.05 M) at pH 5, 7.5 and 9.1 at 23 and

or 18 h. The results were similar at the two temperatures, but higher yields were obtained, as expected, at 55°C. HPLC analysis revealed the presence of the deamination products xanthine and xanthosine (pH 5, 0% yield; pH 7.5, 0.063% yield; pH 9.1, 0.065% yield) in the 55°C run. This same reaction mixture displayed the characteristic doublet HPLC peak of the diastereomeric diols produced from the cyclic condensation of glyoxal and guanosine at *N*-1 and *C*-2 NH<sub>2</sub> (comparison made with authentically prepared material). Yields of this adduct were significantly higher (55°C, 18 h; pH 5, 0.8%; pH 7.5, 2.2%; pH 9.1, 1.7%) than those of xanthosine. Chung and Hecht (1985) have shown this same type of adduct to form from the incubation of NHMOR and deoxyguanosine.



Comparison of the HPLC chromatogram of our reaction mixture with that produced from the reaction of *N*-butyl-*N*-nitrosoacetamide with guanosine revealed a number of peaks in common. This suggested that products derived from the reactions of the butyldiazonium ion were present. Further comparison and spiking experiments utilizing authentically prepared *N*7-1-butylguanosine revealed its presence (0.0018% at pH 9.1, 55°C, 18 h; 0.0087% at pH 7.5), and the identification of other adducts is under way.

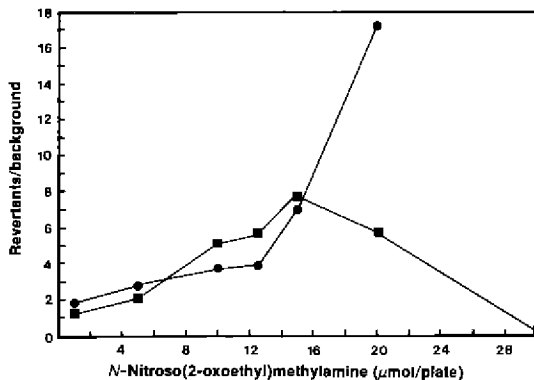
Only traces of these compounds were found at pH 5. The relatively low yield of butylated guanosines can be attributed in part to the diverse chemistry of the butyl diazonium ion on the one hand and to ring-opening reactions of the adduct on the other.

To determine how readily  $\alpha$ -nitrosoaminoaldehydes might be produced from their corresponding environmentally or metabolically-derived alcohols, we have investigated the horse-liver alcohol dehydrogenase oxidation of a series of alkylnitrosoaminoethanols and found all of them to act as substrates for this enzyme. The rates were determined spectrophotometrically by observing NADH concentration changes. Thus, the  $K_m$  (mM) and  $V_{max}$  (mM/min) for NDELA and NBELA are, respectively,  $10^{-1}$ ,  $1.63 \times 10^{-4}$  and  $3.9 \times 10^{-3}$ ,  $1.2 \times 10^{-4}$ . The relative rates of oxidation are: NDELA, 1; NBELA, 14.4; *N*-nitrosomethylethanolamine, 19.2; and ethanol, 3125. The oxidation products were shown to be the corresponding  $\alpha$ -nitrosoaminoaldehydes, by trapping them as their semicarbizones which were compared chromatographically with those produced from the authentic aldehydes. The oxidation was shown to be reversible by demonstrating the horse-liver alcohol dehydrogenase/NADH reduction of several  $\alpha$ -nitrosoaminoaldehydes. Under conditions of excess NADH, the enzymatic reduction of the aldehyde to the alcohol is much faster than the corresponding oxidation in the presence of excess NAD. An interesting feature of this redox reaction was revealed by examination of the reduction of *N*-nitroso(2-oxoethyl)methyl-

amine (NOEMA). This aldehyde is constituted of a 84:16 mixture of Z:E isomers, but the Z isomer is reduced much more rapidly, if not exclusively, to Z-N-nitroso(2-hydroxyethyl)-methylamine. This suggests that the Z-alcohol/aldehyde pair is a much better substrate for the enzyme than their E-counterparts.

The chemical properties of  $\alpha$ -nitrosaminoaldehydes suggest that, like NHMOR, they should be directly-acting mutagens. In order to test this hypothesis, the mutagenicity of NOEMA, NBOEA and N-nitrobutyl(2-oxoethyl)amine was examined in *Salmonella typhimurium* strains TA100 and TA102. Tests were performed using the liquid pre-incubation modification (20 min, 37°C). Compounds were dissolved in dimethyl sulfoxide (100  $\mu$ L), and concentrations between 0 and 50  $\mu$ mol/plate were used at pH 7.4 without activation. NOEMA clearly exhibited a dose-dependent mutagenic effect, as shown in Figure 1. Toxicity became evident at concentrations above 20  $\mu$ mol/plate. N-Nitrobutyl(2-oxoethyl)amine was slightly mutagenic in TA100 but too toxic to provide good dose-response data; however, a dose-dependent mutagenic effect was detected in TA102 (three-fold increase in the number of revertants at 20  $\mu$ mol/plate). This compound was toxic above 200  $\mu$ mol/plate in TA102. NBOEA virtually eradicated the colonies in both strains at concentrations as low as 1  $\mu$ mol/plate. No mutagenic effect could be detected in the range of 0.05-0.5  $\mu$ mol/plate. This compound was also tested by the plate incorporation method, and a slight mutagenic effect was detected at 0.5  $\mu$ mol/plate. These data clearly demonstrate biological properties for  $\alpha$ -nitrosaminoaldehydes that are consistent with their chemical lability and unusual ability to interact with nucleosides in several different ways. While the mutagenicity data are at a preliminary stage and further work is underway, it is obvious that the chemical observations indicate at least three mechanisms for the unusual directly-acting mutagenicity of NHMOR, NOEMA and N-nitrobutyl(2-oxoethyl)amine. The data also provide a mechanism for the alcohol dehydrogenase-mediated mutagenesis of NDELA, which must involve NHMOR. Recent data, however, indicate a higher degree of complexity in the intact animal.

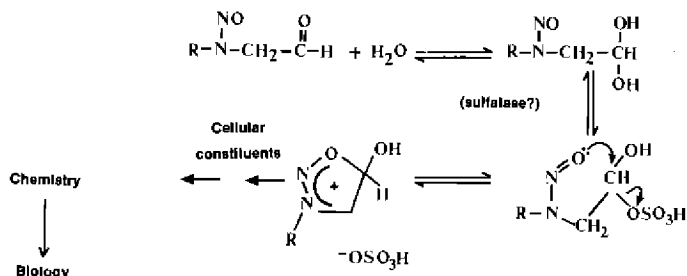
**Fig. 1. Dose-response mutagenesis of NOEMA in *S. typhimurium* TA100 (■) and TA102 (●)**



By measuring DNA strand breaks produced *in vivo* by a series of metabolically related nitrosamines as a function of a sulfotransferase enzyme inhibitor concentration, Sterzel and Eisenbrand (1986) have shown that strand breaks produced by NDELA are completely inhibited. In contrast, strand breaks induced by either NHMOR or N-nitrosoethylethanolamine are only partially blocked by the sulfotransferase inhibitor. They have proposed that NDELA activation requires alcohol dehydrogenase-catalysed oxidation to the aldehyde (which forms the hemiacetal NHMOR), and that this process is followed by sulfate conjugation of the OH group to produce a reactive carcinogen. Eisenbrand and colleagues also suggest that NDELA may be activated by sulfate conju-

gation, according to the Michejda hypothesis (Koepke *et al.*, 1979). On the basis of data from several laboratories, we believe that the  $\alpha$ -nitrosaminoaldehyde hydrates could be further activated by sulfate conjugation followed by neighbouring group attack, as shown in Scheme 3.

Scheme 3



In aqueous solution at pH 7, NOEMA exists to the extent of 97% as a 45:55 mixture of Z and E isomeric 1,1-diols. The Z:E ratio for the unchanged aldehyde (3%) is 84:16, while *N*-nitroso(2-hydroxyethyl)methylamine has a Z:E ratio of 34:66. A similar phenomenon is seen for the more bulky NBOEA (diol:aldehyde = 94:6; Z:E - diol = 65:35 and only the Z aldehyde can be observed in D<sub>2</sub>O). As shown by other authors, the aldehyde produced from NDELA exists as its cyclic hemiacetal NHMOR. These observations, while seeming somewhat esoteric, could have important consequences for the biological activity of these compounds. We first call attention to the fact that the Z:E ratio is significantly altered towards the E isomer in the hydrate. The Z:E equilibrium is kinetically slower than the hydrate-aldehyde equilibrium. We have found the E isomer of NOEMA to be much less reactive toward transnitrosation than its Z counterpart. The Z:E ratio in the aldehydes is unusually biased toward the Z isomer, which suggests an attractive interaction between the carbonyl carbon and the nitroso oxygen, which may effect the reactivity of these compounds. The hydrate and hemiacetal structures could provide, on the one hand, a means of chemically stabilizing these compounds and facilitating their transport and, on the other hand, enable reactions through the OH groups such as conjugation which could either stabilize or predispose the nitrosamine to rapid reaction with cellular components.

### Conclusion

The data summarized above, including that from other laboratories, provide strong evidence that nitrosamino ethanols can be activated to carcinogens through enzymatic oxidation to  $\alpha$ -nitrosamino aldehydes. The chemistry of  $\alpha$ -nitrosamino aldehydes reveals how they could produce damage within the cell through the spontaneous generation of diazonium ions and transnitrosation reactions, which appear to be coupled to the formation of mutagenic glyoxal or its imines. The use of guanosine as a probe has shown that  $\alpha$ -nitrosaminoaldehydes result in deamination, alkylation and glyoxal adduct formation. Any one of these processes could result in mutagenesis. It is clear that the aldehydes can be formed from the liver alcohol dehydrogenase-catalysed oxidation of the  $\beta$ -nitrosamino alcohols and that they are directly-acting mutagens. It is likely that further studies on  $\alpha$ -nitrosaminoaldehydes will reveal a more complex set of interactions with cellular material. Nevertheless, the evidence at hand strongly favours the existence of another pathway for nitrosamine carcinogenic activation which does not require  $\alpha$ -hydroxylation.

# DIRECTLY-ACTING MUTAGENS FORMED FROM N-NITROSO-N-(FORMYLMETHYL)ALKYLAMINES

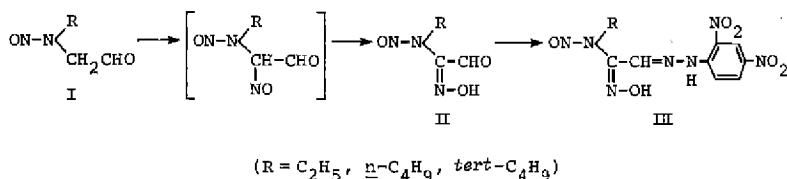
E. Suzuki, M. Osabe, M. Mochizuki<sup>1</sup> & M. Okada

Tokyo Biochemical Research Institute, Takada 3-41-8, Toshima-ku,  
Tokyo 171, Japan

Directly-acting mutagens formed from *N*-nitroso-*N*-(formylmethyl)alkylamines (I) were isolated and identified as *N*-nitroso-*N*-alkyl-1-hydroxyimino-2-oxoethylamines (II). Their structures were elucidated on the basis of nuclear magnetic resonance spectra and confirmed by leading to their crystalline 2,4-dinitrophenylhydrazone. II (alkyl = ethyl and *n*-butyl) were strongly mutagenic to *Salmonella typhimurium* TA1535 and *Escherichia coli* WP2 *hcr*<sup>-</sup> without metabolic activation, while II with a *tert*-butyl group was not mutagenic. The formation of II from I is considered to proceed by the nitrosation of I, indicating a possible involvement of a formylmethyl metabolite in the carcinogenesis of nitrosamines with a 2-hydroxyethyl group.

In addition to the generally accepted  $\alpha$ -hydroxylation pathway for the metabolic activation of nitrosamines, an alternative pathway involving  $\beta$ -hydroxylation has been proposed (Michejda *et al.*, 1981; Airoidi *et al.*, 1984; Loepky *et al.*, 1984; Sterzel & Eisenbrand, 1986). We reported that *N*-nitroso-*N*-(formylmethyl)butylamine (NFMBA) (I; R = *n*-butyl) is chemically unstable and, upon standing at room temperature, produces directly-acting mutagen(s) in the Ames' assay (Suzuki & Okada, 1979). This paper is concerned with the isolation and structural elucidation of the mutagens produced from NFMBA and its alkyl homologues (I) and their biological activities (Fig. 1).

Fig. 1. Formation of *N*-nitroso-*N*-alkyl-1-hydroxyimino-2-oxoethylamines (II) from *N*-nitroso-*N*-(formylmethyl)alkylamines (I)



<sup>1</sup> Present address: Kyoritsu College of Pharmacy, Shibakoen 1-5-30, Minato-ku, Tokyo 105, Japan



**Isolation and identification of directly-acting mutagens, II produced from I**

Diethylacetals of I were synthesized according to the method described previously (Suzuki & Okada, 1979). *N*-Nitroso-*N*-(formylmethyl)ethylamine (NFMEA) was prepared by stirring a mixture of its diethyl acetal in 1N hydrochloric acid for 1 h. NFMBA and *N*-nitroso-*N*-(formylmethyl)-*tert*-butylamine (NFMtBA) were obtained similarly from the corresponding diethyl acetal.

The directly-acting mutagens (II) were isolated as follows from a solution (100 mg/ml) of I (NFMEA, NFMBA, NFMtBA) in methyl acetate after standing at room temperature for several days with an occasional check by thin-layer chromatography: (i) chromatography on silica-gel column (elution with a mixture of hexane:ether:chloroform with a ratio of 4:3:2 or 3:3:2) and then (ii) on Sephadex LH-20 column by elution with a mixture of chloroform:acetonitrile (9:1); the fraction containing II was concentrated under reduced pressure without dryness and was used for mutation assay of II; (iii) derivatization of II with 2,4-dinitrophenylhydrazine in acetonitrile was carried out in the usual way to give 2,4-dinitrophenylhydrazone (III) as yellow needles. R = ethyl, m.p. 132, ~ 3°C (decomposition); R = *n*-butyl, m.p. 119°C (decomposition); R = *tert*-butyl, m.p. 137.5°C (decomposition). On the basis of the nuclear magnetic resonance (Table I) and infrared spectra, and on elemental analyses, the structures of II and III were elucidated.

**Table 1. Nuclear magnetic resonance (NMR) spectra<sup>a</sup> of *N*-nitroso-*N*-alkyl-1-hydroxyimino-2-oxoethylamines (II) and their 2,4-dinitrophenylhydrazones (III)**

II ( $\delta$ ppm)			III ( $\delta$ ppm)		
R	CHO	N-CH <sub>2</sub> <sup>b</sup> or N-C(CH <sub>3</sub> ) <sub>3</sub>	R	CHO	N-CH <sub>2</sub> or N-C(CH <sub>3</sub> ) <sub>3</sub>
C <sub>2</sub> H <sub>5</sub> <sup>b</sup>	9.26 9.54 (s)	3.71 (q) 4.33 (q)	C <sub>2</sub> H <sub>5</sub> <sup>c</sup>	8.34 (s) 8.59 (s)	3.88 (q) 4.51 (q)
<i>n</i> -C <sub>4</sub> H <sub>9</sub> <sup>b</sup>	9.37 (s) 9.67 (s)	3.73 (l) 4.32 (l)	<i>n</i> -C <sub>4</sub> H <sub>9</sub> <sup>c</sup>	8.33 (s) 8.58 (s)	3.90 (t) 4.47 (t)
<i>tert</i> -C <sub>4</sub> H <sub>9</sub> <sup>b</sup>	9.40 (s)	1.52 (s)	<i>tert</i> -C <sub>4</sub> H <sub>9</sub> <sup>d</sup>	8.07 (s)	1.68 (s)

<sup>a</sup>NMR spectra were taken in <sup>b</sup>CD<sub>3</sub>CN, <sup>c</sup>CD<sub>3</sub>COCD<sub>3</sub>, and <sup>d</sup>CD<sub>3</sub>OD at 60 MHz. Chemical shifts are expressed in  $\delta$  (parts per million) with tetramethylsilane as an internal standard; s, singlet; t, triplet; q, quartet

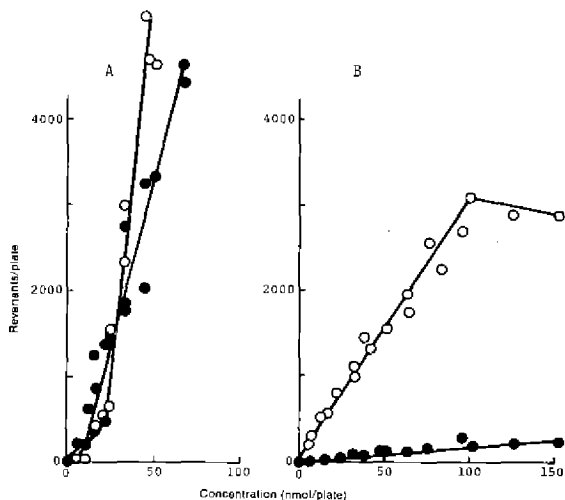
Of the three *N*-nitroso-*N*-alkyl-1-hydroxyimino-2-oxoethylamines (II; R = ethyl, *n*-butyl, *tert*-butyl), the *tert*-butyl compound was stable (crystalline substance, m.p. 104°C, decomposition) and gave 2,4-dinitrophenylhydrazone in nearly 100% yield. Ethyl and *n*-butyl compounds were unstable without solvent, and their content in solution was determined by leading to the 2,4-dinitrophenylhydrazone (III). The yield of II from I varied from 8-18%.

**Mutagenicity of II**

Mutagenicity tests were carried out according to the method reported previously, in the absence (Mochizuki *et al.*, 1979) and in the presence (Yahagi *et al.*, 1977) of

rat-liver 9000 × g supernatant (S9 mix), using *Salmonella typhimurium* TA1535 and *Escherichia coli* WP2 *hcr*<sup>-</sup>. Ethyl and butyl compounds (II, R = ethyl and *n*-butyl) were strongly mutagenic to *S. typhimurium* TA1535 and to *E. coli* WP2 *hcr*<sup>-</sup> without S9 mix (Fig. 2), while the *tert*-butyl compound (II, R = *tert*-butyl) was not mutagenic to either bacterial strain with or without S9 mix.

**Fig. 2. Mutagenicity of II in the absence of S9 mix in *S. typhimurium* TA1535 (○) and *E. coli* WP2 *hcr*<sup>-</sup> (●)**



A, alkyl = ethyl; B, alkyl = *n*-butyl. Data from three different experiments are shown.

under the same experimental conditions (Okada & Hashimoto, 1974; Okada *et al.*, 1976). On the basis of these findings, I, the obligatory metabolic intermediates situated between the 2-hydroxyethyl and carboxymethyl nitrosamines, are suspected to be involved in the carcinogenicity of nitrosamines with a 2-hydroxyethyl group. Thus, NFMBA, *N*-nitroso-*N*-(formylethyl)butylamine and *N*-nitroso-*N*-(formylpropyl)butylamine were synthesized. Only NFMBA was chemically unstable and, upon standing at room temperature, produced directly-acting mutagen(s) in the Ames' assay (Suzuki & Okada, 1979).

The directly-acting mutagens formed from NFMEA and NFMBA upon standing in solution were isolated and identified as II (R = ethyl and *n*-butyl), which are considered to be formed by nitrosation followed by subsequent rearrangement leading to the oxime (II) (Fig. 1). In view of the finding of Loepky *et al.* (1984) that  $\alpha$ -nitrosamino aldehydes readily transfer their nitroso group, and of the formation of II (R = *tert*-butyl) by nitrosation of NFMtBA, the nitrosating agents involved here are presumed to be NFMEA and NFMBA themselves.

#### Nitrosation of *N*-nitroso-*N*-(oxoalkyl)butylamines

In order to examine whether similar *N*-nitroso-*N*-alkyl-1-hydroxyimino-2-oxoalkylamine-type compounds are produced from other nitrosamines with a 2-oxoalkyl group,

#### Formation of II (R = *tert*-butyl) by nitrosation of NFMtBA (I, R = *tert*-butyl)

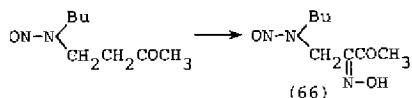
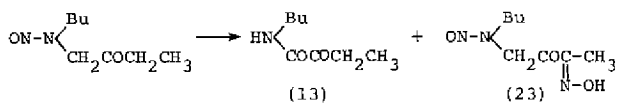
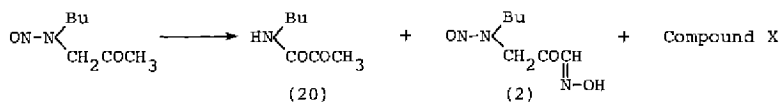
NFMtBA (produced from its diethyl acetal in 0.1N hydrochloric acid) was treated with sodium nitrite to give II (R = *tert*-butyl) in 6% yield after chromatographic purification of the product on a silica-gel column. Nitrosation of NFMtBA in methyl acetate with nitrosyl chloride in chloroform also yielded II (R = *tert*-butyl).

#### Activation of *N*-nitroso-*N*-(2-hydroxyethyl)alkylamines

*N*-Nitroso-*N*-(2-hydroxyethyl)-alkylamines (alkyl = ethyl and *n*-butyl) are potent carcinogens which induce tumours of the liver and oesophagus in rats; however, their principal urinary metabolites, *N*-nitroso- $\alpha$ -(carboxymethyl)-alkylamines are not carcinogenic

*N*-nitroso-*N*-(2-oxopropyl)butylamine (NOPBA) and *N*-nitroso-*N*-(2-oxobutyl)butylamine (N2OBBA) were treated with nitrosyl chloride. The products identified are shown in Figure 3, together with those produced from a nitrosamine with a 3-oxoalkyl group, *N*-nitroso-*N*-(3-oxobutyl)butylamine (N3OBBA). The  $\beta$ -oxo nitrosamines, NOPBA and N2OBBA, are not chemically stable, decomposing upon standing at room temperature, while the  $\gamma$ -oxo nitrosamine N3OBBA is stable. None of the products was mutagenic except Compound X, whose structure has not yet been elucidated.

**Fig. 3. Products formed from *N*-nitroso-*N*-(oxoalkyl)alkylamines by treatment with nitrosyl chloride**



Bu, *n*-C<sub>4</sub>H<sub>9</sub>; percentage yield in parentheses

## ENZYMATIC MECHANISMS IN THE METABOLIC ACTIVATION OF *N*-NITROSODIALKYLAMINES

C.S. Yang, C. Patten, M.J. Lee, M. Li, J.S.H. Yoo, J. Pan & J. Hong

*Department of Biochemistry, University of Medicine and Dentistry  
of New Jersey, New Jersey Medical School, Newark, NJ, USA*

The metabolism of several *N*-nitrosodialkylamines was studied using rat liver microsomes and purified cytochrome P450 isozymes in a reconstituted monooxygenase system. With purified acetone/ethanol-inducible cytochrome P450 (P450<sub>ac</sub>), high *N*-nitrosodimethylamine (NDMA) demethylase activity was observed. Cytochrome b<sub>5</sub> was also involved in NDMA metabolism by decreasing the  $K_m$  of NDMA demethylase. A close relationship between the demethylation and denitrosation of this substrate was observed. P450<sub>ac</sub> was also active in the metabolism of *N*-nitrosoethylmethylamine (NEMA), but was less active than phenobarbital-inducible cytochrome P450 (P450<sub>b</sub>) in the metabolism of *N*-nitrosobutylmethylamine (NBMA), especially in catalysing the debutylation reaction. Similar substrate specificity was demonstrated with liver microsomes from rats treated with other inducers. With different P450 isozymes and microsomes, a close relationship between metabolism and activation of nitrosamines to mutagens to V79 cells was demonstrated. DNA alkylation by NDMA *in vitro* was correlated with the rate of metabolism of these compounds, whereas DNA alkylation *in vivo* was more complex and was dose-dependent. The work demonstrates the importance of knowledge of the substrate specificity of cytochrome P450 isozymes in understanding the mechanisms of the metabolic activation of nitrosamines.

Although NDMA was shown to be metabolized by P450 more than ten years ago (Czygan *et al.*, 1973; Guengerich, 1977), the enzymology of nitrosamine metabolism is not completely understood. Previous results from our laboratory suggest that a specific P450 isozyme, inducible by ethanol, acetone, fasting and other factors, is very active in the metabolism of this nitrosamine (Peng *et al.*, 1982; Tu & Yang, 1983; Tu *et al.*, 1983; Yang, C.S. *et al.*, 1984). Recently, this form of P450 (referred to as P450<sub>ac</sub>) has been purified to homogeneity. The activity of P450<sub>ac</sub> in catalysing nitrosamine metabolism was studied together with that of other P450 isozymes. The present communication deals with enzyme specificity in the metabolism of several *N*-nitrosodialkylamines, as well as the relationship between the metabolism and the activation of these compounds to alkylating agents and mutagens.

### Properties of P450<sub>ac</sub>

P450<sub>ac</sub> was purified from acetone-induced rat-liver microsomes (Tu *et al.*, 1983) by a procedure that is detailed elsewhere (Patten *et al.*, 1986a,b). In brief, the procedure involved chromatography steps, including a lauric acid-AH-Sepharose 4B column (Tu & Yang, 1985), carboxymethyl-Sepharose CL6B column, Pharmacia PBE-94 ion-exchange columns, anti-epoxide hydrolase column, and hydroxylapatite columns. The final P450

preparation is a protein migrating as a single band upon SDS-gel electrophoresis with an estimated  $M_r$  of 52 000. The ferric P450<sub>ac</sub> existed predominantly in the high-spin form at room temperature, and the population of the low-spin form increased upon decreasing the temperature. Judging from the molecular and catalytic properties, P450<sub>ac</sub> is probably similar to P450<sub>j</sub> (Ryan *et al.*, 1985) and is an orthologue to the alcohol-inducible form of P450<sub>LM3a</sub> in rabbit liver (Ryan *et al.*, 1986). This form of P450, which exists in untreated animals, is believed to be responsible for the low  $K_m$  form of NDMA demethylase and for the activation of NDMA *in vivo* (Tu & Yang, 1983; Yang *et al.*, 1985a).

#### Metabolism of nitrosamines by P450<sub>ac</sub> and involvement of cytochrome b<sub>5</sub>

In the presence of NADPH-P450 reductase and phospholipid, P450<sub>ac</sub> catalysed the NADPH-dependent demethylation of NDMA with a turnover number of 14/min (Table 1). In the presence of cytochrome b<sub>5</sub>, the turnover number increased to 28.5/min, and this is the highest ever reported for NDMA demethylase (Patten *et al.*, 1986a). Additional studies showed that maximal NDMA demethylase activity was obtained when cytochrome b<sub>5</sub> was present at equal molarity to P450<sub>ac</sub>. Denatured or apo-cytochrome b<sub>5</sub> was not active in enhancing NDMA demethylase activity. Upon the addition of cytochrome b<sub>5</sub> to the reconstituted system, the apparent  $K_m$  value decreased from 3.19 to 0.35 mM, and the apparent  $V_{max}$  increased from 23.8 to 31.0 nmol/min per nmol (Table 2). To our knowledge, this is the first demonstration that cytochrome b<sub>5</sub> is involved in the metabolism of nitrosamines. Cytochrome b<sub>5</sub> had a similar effect on the apparent  $K_m$  values of P450<sub>ac</sub>-dependent denitrosation of NDMA, decreasing the value from 2.79 to 0.33 mM. The parallel decrease in  $K_m$  values of both reactions by cytochrome b<sub>5</sub> is consistent with our previous suggestion that the demethylation and denitrosation of NDMA may share a common initial step during catalysis (Lorr *et al.*, 1982; Tu & Yang, 1985).

**Table 1. Reconstitution of NDMA demethylase system and the involvement of cytochrome b<sub>5</sub><sup>a</sup>**

Conditions	NDMA demethylase activity (nmol HCHO/min per nmol)
Complete system	28.5
Cytochrome b <sub>5</sub>	14.0
P450	< 0.5
Reductase	< 0.5
Phospholipid	3.6
NADPH generating system	< 0.5
NDMA	< 0.5

<sup>a</sup>The completely reconstituted system consisted of 0.1 nmol P450<sub>ac</sub>, 1000 units NADPH-P450 reductase, 22.5 µg dilauroylphosphatidylcholine, NADPH generating system and 0.1 nmol cytochrome b<sub>5</sub> in a final volume of 0.25 ml. Activity was assayed with a NDMA concentration of 4 mM and an incubation time of 20 min, according to a previously described method (Tu & Yang, 1983).

P450<sub>ac</sub> was also active in catalysing the demethylation of NEMA and of *N*-nitrosomethylbenzylamine (NMBzA), showing turnover values of 1.6 and 2.1 per min, respectively, with 4 mM substrate (Table 3). Cytochrome b<sub>5</sub> stimulated the demethylation of the former substrate (by 25%) but not that of the latter. As expected (Hong & Yang, 1985), P450<sub>ac</sub> catalysed the *para*-hydroxylation of aniline (turnover, 8.0/min with 2 mM aniline), and the activity was slightly enhanced (25%) by cytochrome b<sub>5</sub>. P450<sub>ac</sub> also catalysed the demethylation of benzphetamine and other drug substrates but at rates much lower than those displayed by P450<sub>b</sub>, the major phenobarbital-inducible form in rat liver. P450<sub>b</sub>

**Table 2. Effects of cytochrome  $b_5$  on the kinetic parameters of P450<sub>ac</sub>-dependent metabolism of NDMA<sup>a</sup>**

Product	$K_m$ (mM)		$V_{max}$ (nmol/min per nmol)	
	Without $b_5$	With $b_5$	Without $b_5$	With $b_5$
Formaldehyde	3.19	0.35	23.8	31.0
Nitrite	2.79	0.33	2.3	2.5

<sup>a</sup>Apparent  $K_m$  and  $V_{max}$  values were obtained from the Eadie-Hofstee plot of experimental data obtained from incubations with 0.05, 0.1, 1.0, 2.0 and 4.0 mM NDMA. The linear regression correlation coefficients were 0.996-0.999.

(than two fold) by pretreatment of rats with acetone or ethanol but decreased by pretreatment with phenobarbital or safrole (Table 4). These results are probably due to the fact that NDMA is efficiently metabolized by P450<sub>ac</sub> but less so by P450 isozymes induced by phenobarbital (Tu & Yang, 1985) and, presumably, by those induced by safrole. The metabolism of NEMA followed a similar pattern in response to inducers. The rate of formation of both formaldehyde and acetaldehyde was increased by pretreatment of rats with acetone or ethanol, and the increase appeared to be greater with acetaldehyde than with formaldehyde: the rate of formation of acetaldehyde was about four times greater than that of formaldehyde with all microsomes except those induced by phenobarbital, suggesting that in NEMA the *N*-ethyl group is more efficiently oxygenated than the *N*-methyl group by most of the P450s involved in the metabolism of this nitrosamine. The combined rates for the formation of aldehydes from NEMA were about the same as the rates of formaldehyde formation from NDMA in the different microsomes, as shown in Table 4.

**Table 3. Substrate specificity of P450<sub>ac</sub> in a reconstituted system<sup>a</sup>**

Substrate	Without $b_5$	With $b_5$	Ratio ( $b_5$ :no $b_5$ )
<i>N</i> -Nitrosodimethylamine (1 mM)	5.3	22.6	4.26
<i>N</i> -Nitrosomethylethylamine (4 mM)	1.6	2.1	1.31
<i>N</i> -Nitrosomethylbenzylamine (4 mM)	2.1	1.9	0.90
Aniline (2 mM)	8.0	10.0	1.25
Benzphetamine (1 mM)	5.1	5.0	0.98
Aminopyrine (1 mM)	1.5	1.4	0.93
Ethylmorphine (1 mM)	2.0	1.7	0.85

<sup>a</sup>Activities are expressed as nmol formaldehyde or *para*-aminophenol formed per min per nmol P450. The concentration of each substrate is indicated in parentheses.

displayed a much higher  $K_m$  of NDMA demethylase but was more effective in the metabolism of *N*-nitrosomethylaniline (Tu & Yang, 1985).

### Metabolism of nitrosamines by different P450 isozymes in microsomes and reconstituted systems

Differences in P450 composition of microsomes are indicated by the finding that the microsomal metabolism of NDMA was enhanced (more-

The microsomal metabolism of *N*-nitrosodiethylamine was also enhanced by pretreatment of rats with acetone or ethanol, but the deethylase activity was lower than the corresponding NDMA demethylase activity. Treatment of rats with phenobarbital or safrole increased the deethylase activity slightly, in contrast to the observed decrease of microsomal NDMA demethylase activity with these two inducers. In the metabolism of NBMA, formation of formaldehyde was increased by pretreatment of rats with acetone or ethanol,

**Table 4. Metabolism of nitrosamines by different types of microsomes<sup>a</sup>**

Treatment of rats	NDMA (formaldehyde)	NEMA		NDEA (acetaldehyde)	NBMA		NMBzA	
		Formaldehyde	Acetaldehyde		Formaldehyde	Butyraldehyde	Formaldehyde	Benzaldehyde
Untreated	2.25	0.44	1.55	1.14	1.23	1.52	0.69	1.59
Acetone	4.63	0.75	3.35	2.40	1.88	2.11	0.75	1.66
Ethanol	6.01	0.77	4.48	2.99	2.03	1.38	0.69	1.34
Phenobarbital	0.80	0.23	0.52	1.37	1.16	3.32	0.80	2.71
Safrole	1.86	0.28	1.13	1.31	1.24	2.93	1.33	3.22

<sup>a</sup>Microsomes were prepared from Sprague-Dawley rats (body weights, 80-90 g) treated with inducers according to procedures described previously (Hong & Yang, 1985). For the treatment with safrole, rats were injected at a dosage of 150 mg/kg daily for three days before sacrifice. Formaldehyde produced from NDMA was determined using a modified Nash reagent (Tu & Yang, 1983). The aldehydes formed from other nitrosamines were determined as 2,4-dinitrophenylhydrazone derivatives by high-performance liquid chromatography according to Farrelly (1980). The activity was expressed as nmol aldehyde produced per min per nmol P450. NDEA, *N*-nitrosodiethylamine

and the formation of butyraldehyde was markedly increased by pretreatment with phenobarbital or safrole. These results suggest that phenobarbital- and safrole-inducible P450 isozymes are efficient in catalysing  $\alpha$ -oxygenation of the *N*-butyl group of NBMA. These P450 isozymes also appeared to be efficient in catalysing the  $\alpha$ -oxygenation of the *N*-benzyl group of NMBzA, and safrole-induced microsomes were more active than control microsomes in catalysing the demethylation of NMBzA; however, pretreatment of rats with acetone or ethanol did not enhance the microsomal metabolism of NMBzA (Table 4). Pretreatment of rats with 3-methylcholanthrene decreased the microsomal metabolism of all nitrosamines studied (data not shown). This pattern of enzyme specificity was also observed with P450 isozymes purified from rabbit liver, in that (i) P450<sub>LM3a</sub> (ethanol-inducible) was more active than other isozymes in the metabolism of NDMA and NEMA, and (ii) P450<sub>LM2</sub> (phenobarbital-inducible) was more active than P450<sub>LM3a</sub> in the demethylation of NBMA, NMBzA and *N*-nitrosomethylaniline (Yang *et al.*, 1985b). P450<sub>b</sub> (phenobarbital-inducible form in rat liver) was also shown to catalyse the debutylation and debenzoylation of NBMA more efficiently than P450<sub>ac</sub> (data not shown).

#### Enzyme specificity in the activation of nitrosamines

Conflicting results have been obtained concerning the role of P450 in the metabolic activation of NDMA and other nitrosamines (Phillips *et al.*, 1982; Masson *et al.*, 1983). We believe that many of the controversies are caused by a lack of understanding of the substrate specificity of P450 isozymes and the inhibitor specificity in the metabolism of NDMA. For example, 2-phenylethylamine, 3-amino-1,2,4-triazole and pyrazole, which do not effectively inhibit classical P450-dependent monooxygenase reactions such as benzphetamine demethylase, are efficient inhibitors of P450<sub>ac</sub>-catalysed NDMA demethylation (Tu & Yang, 1985; Yang *et al.*, 1985b). The relationship between P450-dependent NDMA demethylase activity and the metabolic activation of NDMA was demonstrated using DNA alkylation and mutagenesis in V79 cells as endpoints. Induction of NDMA demethylase with ethanol, acetone or isopropanol resulted in an increase in the NDMA-dependent formation of

O<sup>6</sup>-methylguanine and N<sup>7</sup>-methylguanine in experiments *in vitro*. In studies *in vivo*, however, increased DNA alkylation was observed with a high dose (75 mg/kg) but not with low doses of NDMA ( $\leq 25$  mg/kg), possibly due to the kinetics of the activation process (Hong & Yang, 1985). In the metabolism of NDMA, P450<sub>ac</sub> displays a low  $K_m$  whereas P450<sub>b</sub> displays a very high  $K_m$  value. This property was demonstrated clearly in the activation of NDMA to a mutagen for V79 cells (Yoo & Yang, 1985). In other studies, activation of nitrosamines to mutagens by different types of microsomes was also correlated with metabolic activities.

In this work, we hope that we have demonstrated the importance of knowledge of the substrate specificity of P450 isozymes in understanding the mechanisms of the metabolism and activation of NDMA and other nitrosamines.

#### **Acknowledgements**

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# MECHANISM AND CONTROL OF DENITROSION OF *N*-NITROSODIMETHYLAMINE

H.-J. Haussmann & J. Werringloer<sup>1</sup>

*Department of Toxicology, University of Tübingen,  
Wilhelmstrasse 56, D-7400 Tübingen, Federal Republic of Germany*

The NADPH-dependent microsomal denitrosation of *N*-nitrosodimethylamine (NDMA) has been investigated using a new procedure which was devised for the determination of nitric oxide under aerobic conditions. On the basis of the results obtained with rat-liver microsomes it is concluded that nitric oxide is formed as a precursor of nitrite in a superoxide dismutase (SOD)-insensitive reaction. The enzyme involved in the denitrosation was found to correspond to the cytochrome P450 isoenzyme responsible for the dealkylation of NDMA. The chemical mechanism of the liberation of nitric oxide is proposed to be of an oxidative nature.

The formation of nitrite during the NADPH-dependent microsomal metabolism of *N*-nitrosamines is considered to be indicative of a metabolic pathway leading to inactivation of this class of carcinogens (Appel *et al.*, 1980). This assumption is based on the incompatibility of the formation of nitrite and the liberation of molecular nitrogen, which results from the dealkylation and subsequent nonenzymatic formation of the ultimate carcinogen (Kroeger-Koepke *et al.*, 1981). In order to define further the enzymatic nature of the denitrosation reaction and to advance the understanding of its control, a new procedure for the determination of nitric oxide has been devised and applied in the present investigation.

## Determination of nitric oxide

The determination of nitric oxide generated during aerobic biotransformation reactions is based on the stoichiometric co-oxidation of nitric oxide and oxyhaemoglobin (oxyHb), which proceeds according to the following equation (Doyle & Hoekstra, 1981):



where metHb is methaemoglobin.

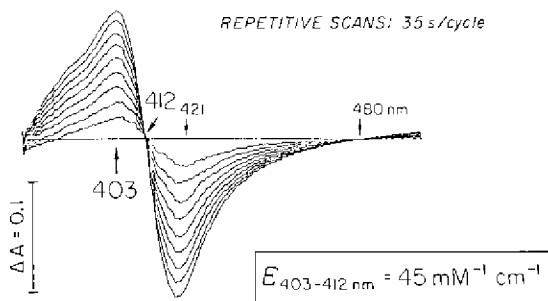
The particular advantage in utilizing oxyHb as a probe for the detection of nitric oxide is the sufficient insensitivity of the above reaction to the presence of molecular oxygen and the amenability of metHb to spectrophotometric quantification.

The applicability of the measurement of this reaction to the analysis of nitric oxide formed during the microsomal metabolism of NDMA has been investigated by difference spectroscopy (Fig. 1). In order to monitor the spectral changes as a function of time, 403 nm and 412 nm were chosen as sample and reference wavelength, respectively. Using ferricyanide as an oxidizing agent, an extinction coefficient of  $45 \text{ mM}^{-1} \text{ cm}^{-1}$  was determined for the quantitative evaluation of the spectral changes observed. The reliability of the assay system proved to be dependent on the additional presence of catalase. This enzyme functions in the protection of haemoglobin against its destruction by hydrogen peroxide,

<sup>1</sup>To whom correspondence should be addressed

which is known to be generated by cytochrome P450 (P450) in an NADPH-dependent reaction (Werringloer *et al.*, 1979). Under the experimental conditions employed, neither catalase nor nitrite, which may be formed independently of nitric oxide, was found to interfere with the metabolic reactions or the assay of nitric oxide, respectively.

**Fig. 1. Conversion of oxyHb to metHb during the microsomal metabolism of NDMA**



The reaction was carried out at 37°C in a medium composed of rat-liver microsomes equivalent to 2  $\mu\text{M}$  P450 (phenobarbital induced), 2.5  $\mu\text{M}$  oxyHb (= 10  $\mu\text{M}$  haem), 25 nM catalase, 50 mM NDMA, 0.2 units/ml isocitrate dehydrogenase, 5 mM isocitrate, 5 mM magnesium chloride, 2 mM 5'-adenosine monophosphate and 0.1 mM NADPH in 50 mM potassium phosphate buffer, pH 7.5.

was found to be limited to the presence of sub-saturating oxyHb concentrations and did not exceed the maximal efficiency of metHb formation under conditions of oxyHb saturation. Accordingly, the SOD-mediated effects are interpreted as indicative of a competitive inhibition by superoxide of the co-oxidation of nitric oxide and oxyHb. On the basis of the results described, it is concluded (i) that nitric oxide is the precursor of nitrite in the denitrosation of NDMA and (ii) that the conversion of nitric oxide to nitrite, generally mediated by molecular oxygen, is enhanced by superoxide, which is known to be formed by P450 (Werringloer *et al.*, 1979). The latter interpretation is consistent with and thus explains the hitherto obscure inhibitory effect of SOD on the formation of nitrite as observed during the microsomal metabolism of various *N*-nitrosamines (Heydt *et al.*, 1982).

Subsequent investigations of the dependence of the denitrosation and dealkylation on the concentration of NDMA revealed a close correspondence of the apparent  $K_m$  values for the formation of nitric oxide, nitrite and formaldehyde (Fig. 2). These results are in full agreement (i) with the above-described identification of nitrite as a secondary product of the denitrosation of NDMA and (ii) with the proposed existence of a linkage between the P450-catalysed reactions resulting in the denitrosation and dealkylation of NDMA (Lorr *et al.*, 1982).

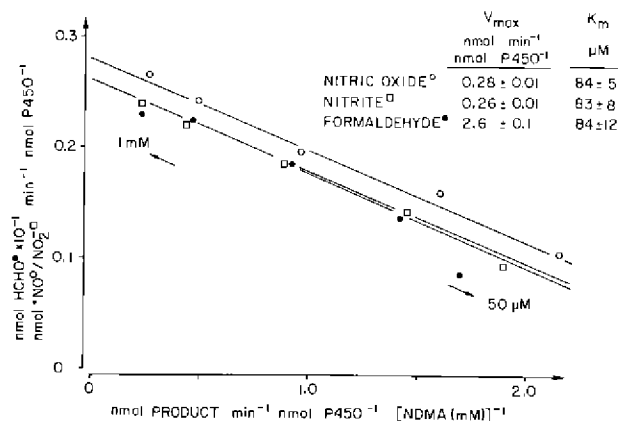
#### Enzymatic nature and control of denitrosation of NDMA

Although the substrate saturation kinetics (see Fig. 2) were found to be consistent with the involvement of a single enzyme in both the denitrosation and dealkylation of NDMA,

#### Formation of nitric oxide and nitrite during the denitrosation of NDMA

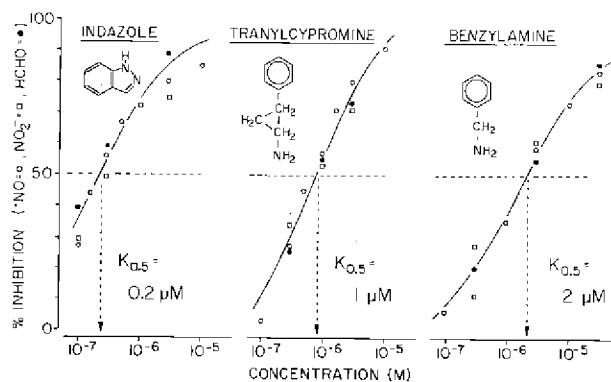
The results illustrated in Figure 1 are indicative of a conversion of oxyHb to metHb during the aerobic metabolism of NDMA. The involvement of nitric oxide as an oxidant in this reaction is indicated by the selectivity of the inhibitory effect of oxyHb on the formation of nitrite, i.e., without effecting the dealkylation of NDMA. The results described are consistent, therefore, with the presumed co-oxidation of nitric oxide and oxyHb, yielding nitrate and metHb as products.

Further investigations revealed a SOD-mediated enhancement of the scavenging of nitric oxide by oxyHb. This enhancement, however,

**Fig. 2. Kinetics of the denitrosation and dealkylation of NDMA**

The experimental conditions were as described in the footnote to Figure 1, except that liver microsomes equivalent to 1 μM P450 (isopropanol induced) were used. Further, oxyHb was omitted from the incubation media employed for the measurement of nitrite and formaldehyde formation. Product analysis was carried out colorimetrically according to the method of Bratton and Marshall (1939) and Werringloer *et al.* (1979), respectively.

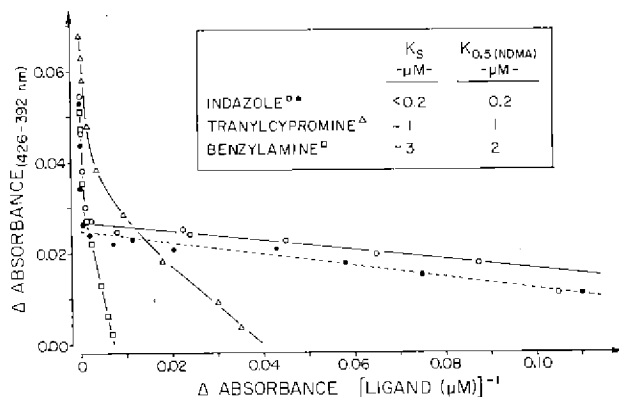
and 4), it is concluded that in liver microsomes both the denitrosation and the dealkylation are catalysed by a single P450 isoenzyme in the presence of low concentrations of NDMA.

**Fig. 3. Inhibition of the denitrosation and dealkylation of NDMA**

The experimental conditions were as described in the footnote to Figure 2, except that the NDMA concentration was kept constant at 1 mM.

further evidence was sought to substantiate this interpretation. The studies carried out in this regard not only confirmed the high affinity of the isopropanol-inducible P450 for NDMA (Yang *et al.*, 1985a; and Fig. 2), but, in addition, revealed its high affinity for a unique class of nitrogenous compounds which are known as inhibitors of the monoamine oxidase and of the dealkylation of NDMA (Lake *et al.*, 1982b). On the basis of the correspondence of the compound-specific inhibition patterns obtained for the formation of nitric oxide, nitrite and formaldehyde (Fig. 3), as well as of the concentrations found to be sufficient for half-maximal inhibition and ligand saturation of this particular enzyme (Figs 3

The chemical mechanism of the denitrosation, however, remains to be defined. The hypothesis that liberation of nitric oxide results from reductive reactions (Appel & Graf, 1982) requires the formation of secondary amines as complementary products; yet, methylamine rather than dimethylamine has been identified as a metabolite of NDMA (Grilli & Prodi, 1975). As an alternative, it is suggested that nitric oxide is liberated in an oxidative reaction *via* aminium cation radicals which are formed upon electron abstraction as initial intermediates in

**Fig. 4. Kinetics of the interaction of P450 and of the inhibitors of the metabolism of NDMA**

The extent of the interaction was determined by difference spectroscopy (Jefcoate, 1978). The reaction media were composed of rat-liver microsomes equivalent to 1  $\mu\text{M}$  P450 (isopropanol induced) in 50 mM potassium phosphate buffer, pH 7.5;  $\bullet$ , indazole binding in the presence of 1 mM NDMA.

P450-catalysed *N*-dealkylations (Hollenberg *et al.*, 1985). These radicals may decay in part, yielding nitric oxide and, as complementary products, alkyliden aminoalkanes rather than secondary amines. This hypothesis is consistent with the detection of methylamine as a metabolite of NDMA, since this primary amine would be formed upon hydrolysis of methylene aminomethane. Hence, the relation between the enzymatic activities leading to an activation or inactivation of this class of carcinogens appears to be dependent on the chemical nature of the substrate and/or the properties of the P450 isoenzyme with a high affinity for *N*-nitrosamines.

#### Acknowledgement

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# POTENTIAL FOR METABOLIC DEACTIVATION OF CARCINOGENIC *N*-NITROSODIMETHYLAMINE *IN VIVO*

L.K. Keefer<sup>1,4</sup>, T. Anjo<sup>1</sup>, Y.-H. Heur<sup>1</sup>, C.S. Yang<sup>2</sup> & B.A. Mico<sup>3</sup>

<sup>1</sup>*Chemistry Section, Laboratory of Comparative Carcinogenesis, National Cancer Institute, Frederick Cancer Research Facility, Frederick, MD 21701, USA;*

<sup>2</sup>*Department of Biochemistry, University of Medicine and Dentistry of New Jersey, New Jersey Medical School, Newark, NJ 07103, USA; and*

<sup>3</sup>*Department of Drug Metabolism, Smith, Kline & French Laboratories, Philadelphia, PA 19101, USA*

Enzymatic cleavage of *N*-nitrosodimethylamine (NDMA) to nitrite (normally representing about 10% of the total metabolism *in vitro*) also produces methylamine in yields roughly equimolar to those of nitrite, suggesting that the 'denitrosation' pathway may be responsible for the previously unexplained detection of methylamine as a urinary metabolite of NDMA and, at least in part, for the recovery of less than stoichiometric amounts of dinitrogen in <sup>15</sup>N-labelling experiments. We have now followed excretion of labelled methylamine by rats receiving <sup>14</sup>C-NDMA as a possible index of the extent of *in-vivo* denitrosation. Correcting for the proportion of labelled methylamine recovered in the urine following its administration under the conditions used for NDMA, 2.5-10% of the NDMA metabolism in Fischer rats appeared to proceed by a methylamine-forming route. The results are consistent with the conclusion that the metabolism of NDMA is best viewed as a competition between two pathways, with denitrosation diverting a significant proportion of the clearance to a presumably deactivating metabolic route at the expense of the activating alkylation pathway responsible for carcinogenesis.

It has been shown recently that metabolic denitrosation of NDMA by rat liver microsomes produces methylamine in yields approximately equimolar with those of nitrite (Keefer *et al.*, 1976). The purposes of this paper are (i) to suggest that denitrosation may accordingly be the 'missing link' of NDMA metabolism capable of rationalizing seemingly anomalous findings from the literature, and (ii) to present preliminary data suggesting that measurement of the urinary excretion of labelled methylamine may be useful in studying the course of this presumably deactivating clearance pathway *in vivo*.

## Metabolism of NDMA

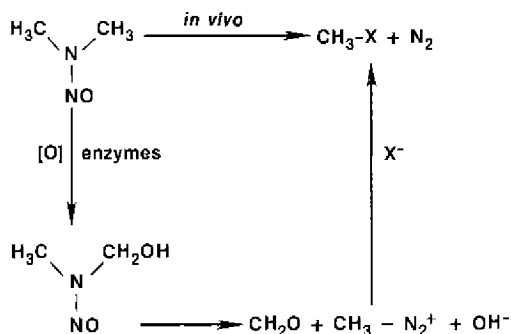
It is widely accepted that NDMA is metabolized as shown in Figure 1, by enzymatic oxygenation of a methyl group followed by the rapid decomposition of the resulting  $\alpha$ -hydroxynitrosamine to formaldehyde, dinitrogen gas and a powerful methylating agent considered to be the ultimately carcinogenic form. Nevertheless,

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<sup>4</sup>To whom correspondence should be addressed

there is growing evidence that a significant proportion of NDMA metabolism proceeds by some route(s) fundamentally different from this. For example, NDMA labelled with  $^{15}\text{N}$  has been found to be metabolized to less than theoretical yields of  $^{15}\text{N}_2$  *in vivo* (Magee, 1980; Micejda *et al.*, 1982) and *in vitro* (Cottrell *et al.*, 1977; Milstein & Guttenplan, 1979; Kroeger-Koepke *et al.*, 1981). Deuterium isotope effect studies have also indicated that a significant elimination route not involving  $\alpha$ -hydroxylation DNA alkylation alone must be operative *in vivo* (Mico *et al.*, 1985; Yang *et al.*, this volume). The data suggest that such metabolism is extensive, accounting for a substantial proportion of total NDMA elimination *in vivo*.

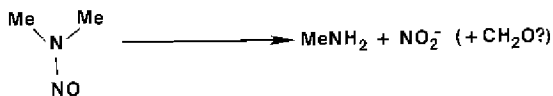
**Fig. 1. Accepted activation pathway resulting in conversion of NDMA to a carcinogenic alkylating agent**



X- represents a cellular nucleophile, such as the oxygen atom at the  $\text{O}^6$  position of a DNA guanine residue.

identified the basic organic product of NDMA denitrosation as methylamine (Keefer *et al.*, 1987). The correspondence in yield between methylamine and nitrite proved to be roughly equimolar, suggesting that either product could serve as an indicator of the extent of the denitrosation process. The overall transformation as presently understood is illustrated in Figure 2.

**Fig. 2. Denitrosation pathway of NDMA metabolism as inferred by Keefer *et al.* (1987)**



denitrosation mechanism operative *in vivo*. It also suggests that excretion of labelled methylamine by rats given  $^{14}\text{C}$ -NDMA could be used to estimate the extent of denitrosation in intact animals.

We have been exploring the hypothesis (Appel *et al.*, 1979b, 1980; Magee, 1980; Schwarz *et al.*, 1980; Lorr *et al.*, 1982; Heydt-Zapf *et al.*, 1983; Arshinov *et al.*, 1984; Appel *et al.*, 1985; Hausmann & Werringloer, 1985; Tu & Yang, 1985; Yang *et al.*, 1985b) that metabolic denitrosation, a pathway shown to cleave the nitrogen-nitrogen bond of nitrosamines *in vitro* (Grilli & Prodi, 1975; Rowland & Grasso, 1975; Appel *et al.*, 1979b, 1980; Schwarz *et al.*, 1980; Schrenk *et al.*, 1981; Appel & Graf, 1982; Heydt *et al.*, 1982; Janzowski *et al.*, 1982b; Lorr *et al.*, 1982; Heydt-Zapf *et al.*, 1983; Appel *et al.*, 1984b; Arshinov *et al.*, 1984; Kuthan *et al.*, 1984; Appel *et al.*, 1985; Hausmann & Werringloer, 1985; Tu & Yang, 1985; Yang *et al.*, 1985b), might be responsible for much or all of the alternative NDMA clearance. As a first step, we

#### Does denitrosation occur *in vivo*?

The identification of methylamine as a product of NDMA denitrosation *in vitro* suggests that the methylamine found by Heath and Dutton (1958) as a urinary metabolite of NDMA may have arisen from a

We now report the first estimates based on this hypothesis. Six male inbred Fischer 344 rats weighing 150-200 g each were cannulated at the jugular vein as described previously (Keefe *et al.*, 1985) and given 1  $\mu\text{mol/kg}$   $^{14}\text{C}$ -NDMA in 1 ml of saline intravenously 24 h after cannulation. Animals were kept in stainless-steel metabolism cages for 24 h after injection. The urine was collected by washing the cages with dilute acetic acid before and after the presence of the animals so as to trap basic products. The urine samples were analysed using an analytical method patterned after that of Heath and Dutton (1958). Aliquots (1 ml each) of acidified urine were spiked with 205 mg unlabelled  $\text{CH}_3\text{NH}_2\text{Cl}$ , evaporated to dryness, and heated with 5 ml of 12 N sulfuric acid for 16 h at  $170^\circ\text{C}$  to hydrolyse methylurea (Dar & Bowman, 1985) and other acid-labile adducts of methylamine. The reaction mixture was cooled, basified and distilled into 5 ml of 5N hydrochloric acid. The receiver contents were evaporated and recrystallized three times from ethanol, giving a sample with a melting-point identical with that of authentic methylamine hydrochloride ( $225\text{-}228^\circ\text{C}$ ). After weighing to determine the recovery, a 1/10 portion was dissolved in 50% ethanol and subjected to scintillation counting. The resulting data permitted calculation of the total injected radioactivity that was excreted as methylamine in the 24-h urines. The values (Table 1) were roughly similar to those observed by Heath and Dutton (1958), or 0.25-1.0% with a mean of 0.7%.

**Table 1. Percent of injected radioactivity excreted as methylamine in 24-h urines of rats dosed intravenously with 1  $\mu\text{mol/kg}$   $^{14}\text{C}$ -NDMA<sup>a</sup>**

Rat	% of $^{14}\text{C}$ excreted
1	0.25
2	1.0
3	0.3
4	0.8
5	1.0
6	0.9
	mean, 0.7%

<sup>a</sup>Specific activity, 54  $\mu\text{Ci}/\mu\text{mol}$

Multiplying the data of Table 1 successively by 2 and 5, the proportion of the NDMA metabolism proceeding by a denitrosation pathway appears to have ranged between 2.5 and 10% (mean, 7%) for the six animals studied.

The results suggest an analytical alternative to the approach used by Arshinov *et al.* (1984) in their important first report on denitrosation of *N*-nitrosodiethylamine (NDEA) *in vivo* (after dosage to rats at 280 mg/kg) and modulation thereof by pretreatment with butylated hydroxytoluene. Following urinary methylamine, instead of plasma nitrite levels, should lead to important advantages, since the analyte is more stable and can be distinguished from that which is endogenously present by the simple device of carbon-labelling the injected nitrosamine. Insight into the sensitivity of the methylamine approach might be gained by comparing the nitrosamine doses used in the two *in-vivo* denitrosation studies: Arshinov *et al.* (1984) administered NDEA at  $> 2\text{ mmol/kg}$ , while we had no trouble measuring excreted radioactivity at NDMA doses three orders of magnitude smaller than that.

These data must be multiplied by a factor of 2 to determine the proportion of the NDMA converted to urinary methylamine, because half of the carbon atoms were lost in the transformation. To determine the percentage yield that these measurements represent, account must be taken of the proportion of the methylamine that, once produced, is further metabolized. We estimated this factor by administering  $^{14}\text{C}$ -methylamine to rats at the same molar dose and under the same conditions as described for NDMA. Consistent with the results of Schwartz (1966), 19% of the  $^{14}\text{C}$ -methylamine was excreted as bound or unbound methylamine 24 h after intravenous bolus administration. Thus, only 1/5 of the methylamine initially present was excreted.

The quantitative conclusions outlined above are presently quite tentative. We cannot be sure that the methylamine production observed after in-vivo metabolism proceeded *via* the denitrosation path, since other mechanisms such as methylation of urea nitrogen followed by hydrolysis could give the same result. Moreover, we have not yet measured the precision of the methylamine assay — a lengthy, multistep procedure that may be associated with a considerable experimental error. It also remains to be determined whether the conditions used in collecting data for Table 2 are relevant to establishing the fate of methylamine produced in the metabolism of NDMA; experiments aimed at refining the answer to this question by studying how urinary methylamine recoveries might vary as a function of dose, route of administration, and other factors are now in progress.

**Table 2. Percent of injected  $^{14}\text{C}$ -methylamine excreted as such in 24-h urines of rats given 1  $\mu\text{mol}/\text{kg}$  intravenously<sup>a</sup>**

Rat	% of $^{14}\text{C}$ excreted
A	18.1
B	17.0
C	19.7
D	22.6
E	20.3
F	18.2
	mean, 19.3%

<sup>a</sup>Specific activity, 49  $\mu\text{Ci}/\mu\text{mol}$

Further studies of the type described above are planned with the hope of developing an increasingly refined understanding of denitrosation as an in-vivo clearance route for NDMA. Such an understanding seems especially desirable in light of growing evidence that a significant flux of this potent carcinogen may be produced in the human body. Particular emphasis will be placed in future work on a search for a possible means of increasing the extent of methylamine generation from NDMA relative to diazonium ion production, because such a shift in metabolism could lead to protection against the cancer risk of NDMA exposures even when the nitrosamine is produced endogenously.

### Conclusions

A plausible mechanistic explanation for the appearance of methylamine as a urinary metabolite of NDMA (Heath & Dutton, 1958) is suggested by the discovery that this amine, rather than dimethylamine, is the basic organic product of enzymatic NDMA denitrosation by liver microsomes. First estimates of the proportion of NDMA metabolism proceeding *via* the methylamine-forming pathway in the intact rat indicate that 2.5-10% of the injected dose followed this clearance route in the animals we studied. The results appear to provide an advantageous basis for probing the competition between 'demethylation' (i.e., the process leading to  $\text{N}_2$  formation, Fig. 1) and 'denitrosation' (Fig. 2) of NDMA *in vivo*, and thus for studying possible means of modulating the relative extents of activation and detoxification of this normally potent carcinogen at low doses relevant to determining the fate of NDMA endogenously produced in the human body.

### Acknowledgements

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Despite the preliminary nature of these first in-vivo measurements, however, it seems clear that the formation of methylamine from NDMA is much more important than the original experiments of Heath and Dutton (1958) might appear to suggest, inasmuch as most of the initial metabolite appears to be eliminated by some route(s) other than urinary excretion. It is crucial that appropriate corrections for this sequential metabolism be applied whenever data on recovery of labelled methylamine from urine are used as the basis for inferences regarding the quantitative course of nitrosamine deactivation *in vivo*.



## SOME ASPECTS OF CYTOCHROME P450-DEPENDENT DENITROSATION OF *N*-NITROSAMINES

K.E. Appel<sup>1</sup>, M. Schoepke<sup>1</sup>, T. Scheper<sup>1</sup>, S. Görsdorf<sup>1</sup>, M. Bauszus<sup>1</sup>,  
C.S. Rühl<sup>1</sup>, R. Kramer<sup>1</sup>, H.H. Ruf<sup>2</sup>, B. Spiegelhalder<sup>3</sup>,  
M. Wiessler<sup>3</sup> & A.G. Hildebrandt<sup>1</sup>

<sup>1</sup>Max von Pettenkofer Institute, Federal Health Office,  
100 Berlin (West) 33; <sup>2</sup>Institute for Physiological Chemistry,  
University of the Saarland, 6650 Homburg/Saar; and

<sup>3</sup>Institute for Toxicology and Chemotherapy, German Cancer Research Centre,  
Im Neuenheimer Feld, 6900 Heidelberg, Federal Republic of Germany

The present paper deals with three aspects of cytochrome P450-dependent denitrosation of *N*-nitrosamines. (1) Nitrate was found in addition to nitrite as a metabolic product of the denitrosation reaction when *N*-nitrosamines were incubated with a microsomal system. This could also be shown when nitric oxide was added to the microsomes. (2) In order to determine the amount of denitrosation *in vivo*, the nitroso group of *N*-nitroso-*N*-methylaniline was labelled with the <sup>15</sup>N isotope and administered to rats; then, the concentrations of <sup>15</sup>*N*-nitrate and <sup>15</sup>-*N*-nitrite in the urine were quantified by measuring the reaction of nitrate and benzene to nitrobenzene. It is estimated from these data that about 33% of the applied dose of <sup>15</sup>*N*-nitroso-*N*-methylaniline is denitrosated *in vivo*. (3) Although *N*-nitrosodiphenylamine (NDPhA) has been classified as a noncarcinogen, recent long-term and short-term studies have cast some doubt. In order to evaluate the mechanism by which NDPhA exerts its possible genotoxic effects, its metabolism was studied *in vitro*, and NDPhA and its metabolites were tested for induction of DNA single-strand breaks in rat hepatocytes and in Chinese hamster V79 cells. One metabolite was identified as diphenylamine; others were suspected to be the 4-hydroxylated derivative and its corresponding quinoneimine. NDPhA caused DNA damage in rat hepatocytes but not in V79 cells. Diphenylamine also gave negative results in V79 cells, but its putative metabolite, diphenylhydroxylamine, induced a significant increase in DNA single-strand breaks. The metabolic capacity of hepatocytes and the chemical nature of NDPhA indicate that denitrosation is the first metabolic step in the biotransformation of NDPhA leading to diphenylamine and diphenylhydroxylamine. As shown by electron spin resonance spectroscopy, diphenylhydroxylamine is autoxidized to the diphenylnitroxide radical. This suggests that diphenylhydroxylamine might be responsible for the observed genotoxic effects of NDPhA, which are possibly produced *via* active oxygen species.

We have suggested, on the basis of mechanistic experiments, that *N*-nitrosamines are denitrosated reductively by the catalytic action of cytochrome P450 (P450). Denitrosation is performed by the transfer of one electron from the haem moiety of P450 to the molecule leading to nitric oxide and the secondary amine (Appel *et al.*, 1980; Appel & Graf, 1982; Appel *et al.*, 1984a,b, 1986). This paper describes further experiments to characterize this pathway of *N*-nitrosamine metabolism.

### Nitrate as a metabolic product formed in addition to nitrite from denitrosation of *N*-nitrosamines

Chemically, both nitrite and nitrate might be formed if free nitric oxide is the intermediary product resulting from denitrosation of nitrosamines in microsomes. In order to prove this supposition, we measured the metabolic formation of both nitrate and nitrite from two *N*-nitrosamines, NDPhA and *N*-nitroso-*tert*-butylmethylamine (NtBMA).

To compare the relationship between the formation of nitrite and nitrate, nitric oxide was also incubated with liver microsomes and an NADPH-regenerating system. Nitrate was determined by three methods: (i) by high-performance liquid chromatography using a Dionex Ion Chromatograph with a coupled conductivity detector, as described by Appel *et al.* (1984a,b); (ii) by using nitrate reductase and detecting the consumption of NADPH when nitrate was reduced to nitrite by the enzyme (the test combination for nitrate determination was obtained from Dr G. Henninger, Boehringer Mannheim) and (iii) by using cadmium reduction. Nitrite was determined by the azo dye method with sulfanilamide and  $\beta$ -naphthylethylenediamine.

Nitrate is formed in addition to nitrite when either NDPhA or NtBMA is incubated with liver microsomes from phenobarbital-pretreated NMRI mice complemented with an NADPH regenerating system (Table 1). The amounts of nitrate measured by the various methods are not always in a similar range. Addition of gaseous nitric oxide to the microsomal suspension also yielded both nitrate and nitrite, both in the presence and absence of the NADPH-regenerating system. The higher amount of nitrate in relation to nitrite in the presence of the NADPH-regenerating system may be due to oxidation of the nitrite to nitrate, e.g., by the attack of hydroxyl radicals (Saul & Archer, 1984). We therefore assume that the metabolic denitrosation of *N*-nitrosamines is underestimated stoichiometrically when only nitrite is determined *in vitro*. The difference in the nitrite/nitrate quotients obtained with NDPhA and NtBMA cannot be explained at the present time.

### Denitrosation of *N*-nitrosomethylaniline *in vivo*: determination by labelling the nitroso group with $^{15}\text{N}$

The denitrosation reaction has been quantified *in vivo* by labelling the nitroso group with the  $^{15}\text{N}$  isotope, since it was assumed that with low doses of *N*-nitrosamines, nitrite/nitrate would not be detectable in urine.

*N*-Methylaniline was nitrosated with  $^{15}\text{N}$ -sodium nitrite to yield  $^{15}\text{N}$ -nitrosomethylaniline. This compound was then dissolved in corn oil and administered to rats at various doses by intraperitoneal injection, and urine samples were taken at 24-h intervals for determination of  $^{15}\text{N}$ -nitrite and  $^{15}\text{N}$ -nitrate (Fig. 1). Most of the  $^{15}\text{N}$ -nitrite/nitrate was detected within 24 h, and only minor amounts were measurable in the 24-48- and 48-72-h intervals. The dose-dependency was linear over a wide range. About 16.5% of the dose was found as nitrite/nitrate in the urine.

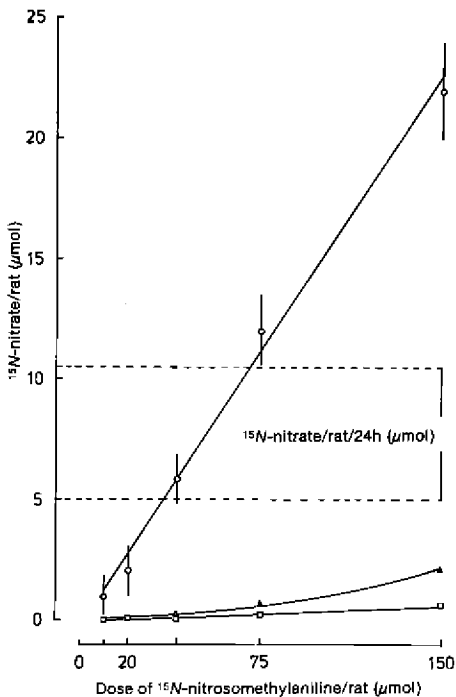
To exclude the possibility that the  $^{15}\text{N}$ -nitrite/nitrate arose from unmetabolized  $^{15}\text{N}$ -nitrosomethylaniline in the urine by reaction with sulfuric acid and hydrogen peroxide, unaltered compound was measured in urine by thermal energy analysis after administration of unlabelled *N*-nitrosomethylamine to rats. Only about 1% of the administered dose was detected, in accordance with the findings of Pylypiw *et al.* (1984).

Table 1. Nitrite and nitrate concentrations in microsomal incubation systems after incubation of NDPPhA, NtMBA and nitric oxide (NO) with liver microsomes from phenobarbital-pretreated NMRI mice (female) with or without an NADPH-regenerating system under aerobic conditions for 30 min<sup>a</sup>

	Ion — HPLC chromatograph			Nitrate reductase			Cadmium reductase		
	NO <sub>2</sub> <sup>-</sup> (μM)	NO <sub>3</sub> <sup>-</sup> (μM)	NO <sub>2</sub> <sup>-</sup> /NO <sub>3</sub> <sup>-</sup>	NO <sub>2</sub> <sup>-</sup> (μM)	NO <sub>3</sub> <sup>-</sup> (μM)	NO <sub>2</sub> <sup>-</sup> /NO <sub>3</sub> <sup>-</sup>	NO <sub>2</sub> <sup>-</sup> (μM)	NO <sub>3</sub> <sup>-</sup> (μM)	NO <sub>2</sub> <sup>-</sup> /NO <sub>3</sub> <sup>-</sup>
NDPhA (1 mM)	140 ± 8	141 ± 51	1.0	167 ± 9	232 ± 19	0.7	109 ± 10	146 ± 8	0.7
NtMBA (1 mM)	157 ± 9	78 ± 38	2.0	155 ± 31	42 ± 6	3.7	75 ± 11	55 ± 7	1.4
NO (0.9 mM) without NADPH	547 ± 55	16 ± 3	34.2	523 ± 67	64 ± 22	8.2	493 ± 51	200 ± 36	2.5
NO (0.9 mM) with NADPH	588 ± 57	127 ± 9	4.6	425 ± 24	204 ± 10	2.1	388 ± 11	301 ± 32	1.3

<sup>a</sup>Nitrite was determined by azo dye formation using β-naphthylethylenediamine and sulfanilamide according to standard procedures. Nitrate was determined by three methods: (i) ion-high-performance liquid chromatography (HPLC), (ii) nitrate reductase by measuring the consumption of NADPH; and (iii) cadmium reduction. Generally, incubations were performed with phosphate buffer pH 7.4, but, when applying the HPLC method for determination of nitrate, Tris-KCl buffer pH 7.4 was used instead. The values represent means from three to five experiments.

**Fig. 1. Quantification of  $^{15}\text{N}$ -nitrite/nitrate in the urine of rats after administration of  $^{15}\text{N}$ -nitrosomethylaniline**



Urine was sampled at 24-h intervals and  $^{15}\text{N}$ -nitrite/nitrate was determined by reaction with benzene and sulfuric acid, in the presence of hydrogen peroxide, to yield  $^{15}\text{N}$ -nitrobenzene, which was then quantified using a mass selective detector, according to the method of Green *et al.* (1982). ○, 0-24 h; △, 24-48 h; □, 48-72 h

mammalian and microbial cells and did not induce transformation in mammalian cells. However, it was shown recently to induce morphological transformation of hamster embryo cells (Schuman *et al.*, 1981) and was mutagenic to *Salmonella typhimurium* TA98 in the presence of norharman (Wakabayashi *et al.*, 1982).

We have tested NDPhA for induction of DNA damage in the alkaline elution assay in rat hepatocytes and Chinese hamster V79 cells, in order to evaluate the mechanism by which NDPhA exerts its toxic effects. The metabolism of NDPhA in liver microsomes was also investigated.

The denitrosation rate *in vivo* must be higher than that indicated by the amounts of  $^{15}\text{N}$ -nitrite/nitrate found in the urine, owing to the fact that nitrite/nitrate is picked up in other metabolic pathways, e.g., urea synthesis products that have not been detected by us. Yoshida *et al.* (1983) studied the recovery of nitrite and nitrate in the urine of rats after intraperitoneal administration: only 53-60% nitrite and 78% nitrate were recovered.

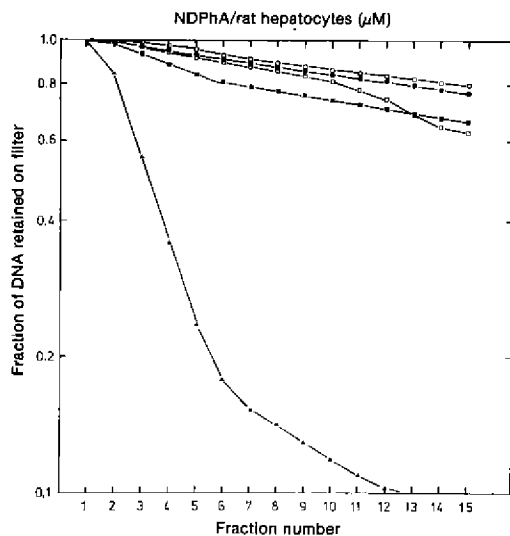
Minor amounts were detected in the faeces or found incorporated in urea, but the remainder was not recovered. When rats were forced to inhale  $^{15}\text{N}$ -nitric oxide (145 ppm, 123 min) and  $^{15}\text{N}$  determined in urine up to 48 h later, recovery was found to be 55% of the inhaled dose (Yoshida *et al.*, 1980, 1983). We assume, therefore, that about 33% of the administered dose of  $^{15}\text{N}$ -nitrosomethylaniline is denitrosated *in vivo*.

#### Denitrosation of *N*-nitrosodiphenylamine: a possible bioactivation pathway

As NDPhA possesses no oxidizable hydrogen in the  $\alpha$ -position, the molecule is not susceptible to the generally accepted oxidative bioactivation pathway of *N*-nitrosamines. On the basis of earlier studies in rats and mice, NDPhA was classified as a noncarcinogen (Argus & Hoch-Ligeti, 1961; Druckrey *et al.*, 1967; Boyland *et al.*, 1968). However, more recent data have shown that it induces transitional-cell carcinomas in the bladder of rats and lung adenomas after skin painting on hairless mice (Cardy *et al.*, 1979; Iversen, 1980). NDPhA failed to induce DNA repair in rat hepatocytes, was nonmutagenic in both

Figure 2 represents the elution profiles of rat hepatocyte DNA after treatment with NDPhA at different doses. With 1 mM NDPhA, a significantly increased elution rate was seen, indicating that either NDPhA itself, or a metabolite, produces DNA single-strand breaks. Addition of 600  $\mu\text{M}$  NDPhA gave the same elution range as the positive control, while 300  $\mu\text{M}$  had no effect, in accordance with the results of Bradley *et al.* (1982). NDPhA was not active in V79 cells, even at higher doses.

**Fig. 2.** Elution profiles of rat hepatocyte DNA after treatment with different doses of NDPhA:  $\circ$ , control;  $\square$ , 500  $\mu\text{M}$  *N*-nitroso-*n*-butylamine (positive control);  $\bullet$ , 300  $\mu\text{M}$  NDPhA;  $\blacksquare$ , 600  $\mu\text{M}$  NDPhA;  $\blacktriangle$ , 1000  $\mu\text{M}$  NDPhA



Each profile represents the mean value of two identically treated samples. Hepatocytes were isolated by a collagenase perfusion technique. The alkaline elution assay was performed according to Kohn *et al.* (1981), with modifications. Isolated hepatocytes were cultured in 5-cm Nunc plastic petri dishes in the same medium as V79 cells. The hepatocytes did not attach to these dishes during the incubation period. Cells were treated with the test compound for 2 h at 37°C.

effects of NDPhA such as carcinogenesis, morphological transformation of hamster embryo cells and DNA single-strand breaks in rat hepatocytes.

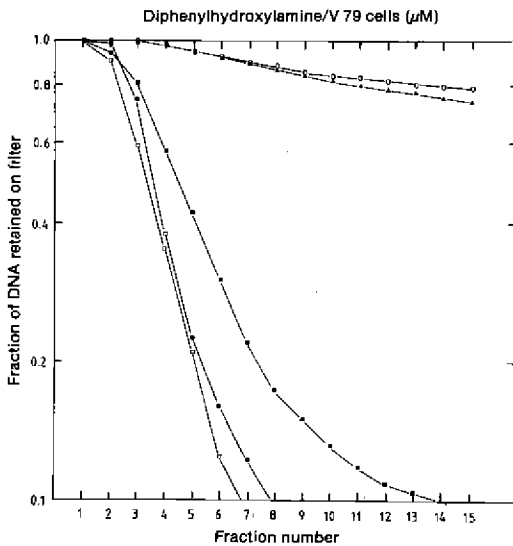
We have not yet been able to detect this metabolite in the high-performance liquid chromatograms after incubation of NDPhA or diphenylamine with microsomes, owing perhaps to its chemical instability. Studies of Alexander *et al.* (1965) indicate that *N*-hydroxylation of diphenylamine might occur. Although those authors were unable to detect

In view of the chemical nature of NDPhA, reductive denitrosation and ring hydroxylation are probably the main metabolic pathways of NDPhA. Therefore, NDPhA was incubated with liver microsomes from NMRI mice pretreated with phenobarbital in order to determine the metabolites resulting from these pathways. Metabolites and unaltered compound were extracted with ethyl acetate and were subjected to high-performance liquid chromatography. Under these conditions, three metabolites were found, one of which represents diphenylamine; the other two metabolites are suspected to be 4-hydroxydiphenylamine and its corresponding quinoneimine. Metabolites with identical retention times and ultraviolet spectra were also found when diphenylamine was incubated with liver microsomes from phenobarbital-pretreated mice. Qualitatively similar results were obtained when NDPhA was administered *in vivo* and the metabolites determined in the urine of rats (Appel *et al.*, 1984b).

*N*-Hydroxylation is recognized as the initial step in the bioactivation of carcinogenic arylamines, although the nature of the ultimate carcinogen — possibly a free radical or a nitrenium ion which induces DNA adducts and DNA damage — is still under debate. Therefore, it may be the *N*-hydroxy derivative of diphenylamine that causes the toxic

this metabolite in the urine of rats or rabbits, oral administration of diphenylamine to a single cat led to considerable formation of methaemoglobin, which might be indicative of the formation of diphenylhydroxylamine. Similarly, after administration of this compound, severe cyanosis was observed in rats, indicating methaemoglobin production (Alexander *et al.*, 1965). We therefore synthesized this putative metabolite and tested it for DNA single-strand break formation in V79 cells. Figure 3 shows that diphenylhydroxylamine is active in inducing DNA single-strand breaks.

**Fig. 3. Elution profiles of Chinese hamster V79 DNA after treatment with different doses of diphenylhydroxylamine (DPhAOH):** ○, control; □, 100  $\mu$ M *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (positive control);  $\Delta$ , 300  $\mu$ M DPhAOH; ■, 600  $\mu$ M DPhAOH; ●, 1000  $\mu$ M DPhAOH



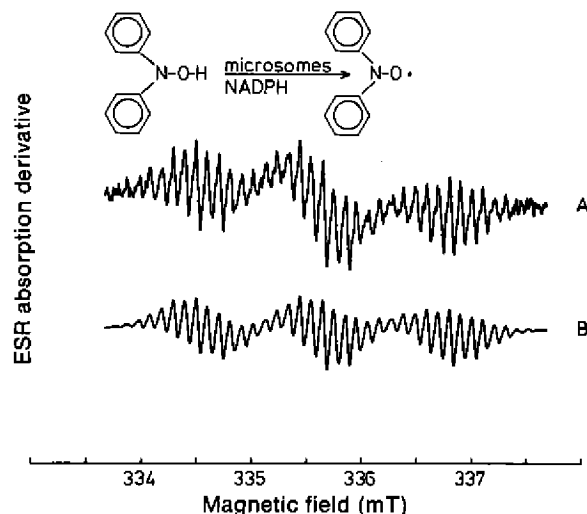
Each profile represents the mean value of two identically treated samples. V79 cells, kindly provided by Dr W. v.d. Hude (Berlin (West), FRG), were grown as a monolayer culture in 25-cm<sup>2</sup> Nunclon flasks (NUNC, Denmark) in Earle's MEM supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 units/ml) and streptomycin (100  $\mu$ g/ml) (all from Biochrom, Berlin (West), FRG) at 37°C, 5% CO<sub>2</sub> and a 100% humidified atmosphere. V79 cell cultures used for the experiment were in exponential growth with a doubling time of 12 h. Cells were treated with the test compound (dissolved in dimethyl sulfoxide) in medium for 2 h at 37°C. After incubation, the cells were detached using trypsin.

The mechanism by which the genotoxic effects are produced by this secondary arylhydroxylamine is not known. Formation of nitrenium ion cannot be excluded. Covalent binding to DNA is unlikely, in view of its probable chemical stability.

A possible explanation is indicated by the electron spin resonance spectrum (Fig. 4) observed after incubation of diphenylhydroxylamine with microsomes. It shows formation of the corresponding nitroxide radical, which is obviously produced by autoxidation rather than by an enzymatic process. Autoxidation of *N*-hydroxy metabolites of carcinogenic arylamines generates reactive oxygen species, like hydrogen peroxide and superoxide anion radicals; and a good correlation has been found between active oxygen formation, convertibility to free radicals and carcinogenic potential of aromatic amines (Stier *et al.*, 1982; Nakayama *et al.*, 1983). Furthermore, it has been shown that DNA lesions are induced in cultured human fibroblasts and mouse FM3A cells by active oxygen species generated from *N*-hydroxy-2-naphthylamine and 3-hydroxyamino-1-methyl-5*H*-pyrido[4,3*b*]-indole (Kaneko *et al.*, 1985; Wakata *et al.*, 1985).

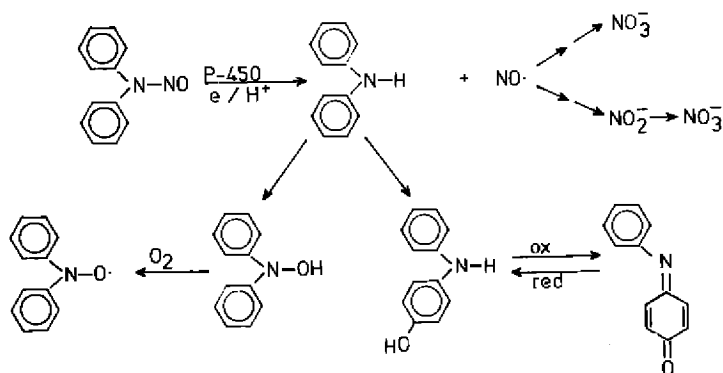
Autoxidation of diphenylhydroxylamine in the cell nucleus, where protective enzymes like superoxide dismutase and catalase are missing, might be the cause of the observed genotoxic effects. Denitrosation of NDPhA would thus represent a bioactivation rather than an inactivation pathway (Fig. 5).

**Fig. 4.** Electron spin resonance (ESR) spectrum obtained after incubation of diphenylhydroxylamine (1 mM) with rat liver microsomes (phenobarbital-pretreated, 24 mg protein/ml; 2.3 nmol P450/mg) and NADPH (1 mM)



After 5 min incubation, the ESR spectrum (A) was recorded (gain,  $4 \times 10^4$ ). After 30 min,  $3 \mu\text{l}$  of an unknown concentration of the diphenylnitroxide radical were added and spectrum (B) was recorded (gain,  $1 \times 10^3$ ). ESR was measured with a Varian E-9 spectrometer at  $22^\circ\text{C}$  (microwave frequency, 9.5 GHz; power, 2 mW; modulation amplitude,  $40 \mu\text{T}$ ). ESR spectra were stored on-line in a digital computer (Data General Nova) for baseline correction and double integration.

**Fig. 5.** Proposed scheme of metabolism of NDPhA



#### Acknowledgements

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# KINETIC ISOTOPE EFFECT ON THE DEMETHYLATION AND DENITROSATION OF *N*-NITROSODIMETHYLAMINE *IN VITRO*

C.S. Yang<sup>1</sup>, D. Wade<sup>1</sup>, T. Anjo<sup>2</sup> & L.K. Keefer<sup>2</sup>

<sup>1</sup>*Department of Biochemistry, University of Medicine and Dentistry of New Jersey, New Jersey Medical School, Newark, NJ 07103, USA; and* <sup>2</sup>*Chemistry Section, Laboratory of Comparative Carcinogenesis, National Cancer Institute, Frederick Cancer Research Facility, Frederick, MD 21701, USA*

Deuteration of *N*-nitrosodimethylamine (NDMA) has been shown to decrease the carcinogenicity of this compound. This result is believed to be due to a kinetic isotope effect on the metabolic activation of this carcinogen, but conflicting views exist concerning whether the isotope substitution affects the  $K_m$  or  $V_{max}$  of the reaction. In order to elucidate the molecular basis of these observations, as well as the mechanisms of the demethylation and denitrosation reactions, the metabolism of NDMA and deuterated NDMA (NDMA-d<sub>6</sub>) was studied using acetone-induced rat-liver microsomes. The demethylation of NDMA displayed a  $K_m$  of 0.06 mM and a  $V_{max}$  of 7.9 nmol/min per mg protein. Deuteration of NDMA increased the  $K_m$  value by five fold but did not appreciably affect the  $V_{max}$ . The denitrosation of NDMA also displayed a  $K_m$  of 0.06 mM, but the  $V_{max}$  was 0.83 nmol/min per mg; deuteration again increased the  $K_{max}$  several fold but had no effect on the  $V_{max}$ . The results indicate that deuteration inhibits the metabolism of NDMA by increasing the  $K_m$  but not the  $V_{max}$  and suggest that there is a close relationship between the demethylation and denitrosation reactions.

Dialkyl nitrosamines, such as NDMA, are potent carcinogens whose organ specificity can be altered by varying the structures of their alkyl groups (Challis & Challis, 1982). One method of changing the structure of an alkyl group is to replace the hydrogen atoms of the group with deuterium. Keefer *et al.* (1973) found that the carcinogenicity of NDMA was reduced by replacing its six protium atoms with deuterium. Swann *et al.* (1983) found a small, probably insignificant, isotope effect on the in-vivo conversion of NDMA to carbon dioxide when NDMA and NDMA-d<sub>6</sub> at 40 mg/kg were administered separately, but a larger effect when they were administered simultaneously. From this result, they concluded that carbon-hydrogen bond cleavage was not the rate-limiting factor in the in-vivo metabolism of NDMA under saturating conditions, but rather that there was free equilibration between unbound NDMA and NDMA in the enzyme-NDMA complex. In addition, they found that, with low doses of NDMA, isotopic substitution produced an effect upon the tissue distribution of DNA alkylation by NDMA. This effect was attributed to a decrease in the extent of first-pass elimination of the deuterated compound. In a recent study of the in-vivo pharmacokinetics of low doses of NDMA, Mico *et al.* (1985) demonstrated an isotope effect on the clearance of NDMA from the blood. As in the study by Swann *et al.* (1983), the effect was more pronounced when the isotopic analogues were



administered simultaneously (Mico *et al.*, 1985). These observations are important in understanding the mechanism of activation of this carcinogen. Nevertheless, the enzymatic basis for the observed isotope effects is not clear. In addition to demethylation, NDMA can be metabolized by cytochrome P450 to form nitrite (Appel *et al.*, 1979a; Appel & Graf, 1982; Lorr *et al.*, 1982; Tu & Yang, 1985; Yang *et al.*, 1985b), but the mechanism of denitrosation is not clear and the effect of deuterium substitution on this reaction is not known.

The study described herein was an attempt to elucidate the molecular basis of the effect of deuteration upon the metabolism of NDMA. Acetone-induced rat-liver microsomes were used as a source of enzyme, and the effects of deuteration were monitored by examining the kinetic parameters associated with the demethylation and denitrosation of NDMA by previously described methods (Appel & Graf, 1982; Tu & Yang, 1985). Treatment of rats with acetone has been shown to induce a cytochrome P450-dependent nitrosamine demethylase (Tu *et al.*, 1983). This activity, which also exists in untreated animals, is believed to be important in the activation of NDMA (Tu & Yang, 1983; Yoo & Yang, 1985). The simultaneous study of demethylation and denitrosation also allows us to elucidate the mechanistic relationship between these two reactions in the metabolism of NDMA.

#### Effect of substrate deuteration on demethylation and denitrosation of NDMA

The existence of multiple  $K_m$  values in the microsomal demethylation of NDMA has puzzled many investigators and complicated many previous studies. The selection of acetone-induced rat-liver microsomes, in which a low- $K_m$  (0.06 mM NDMA) NDMA demethylase is the predominant form of this enzyme, and of low substrate concentrations (from 0.04 to 1.50 mM) for the present study allows us to focus on this form of enzyme with little interference from the high- $K_m$  NDMA demethylase activities. Eadie-Hofstee plots were used to obtain  $K_m$  and  $V_{max}$  values for both the demethylation and denitrosation reactions. In this simple treatment, however, these values can be considered only as apparent  $K_m$  and  $V_{max}$ .

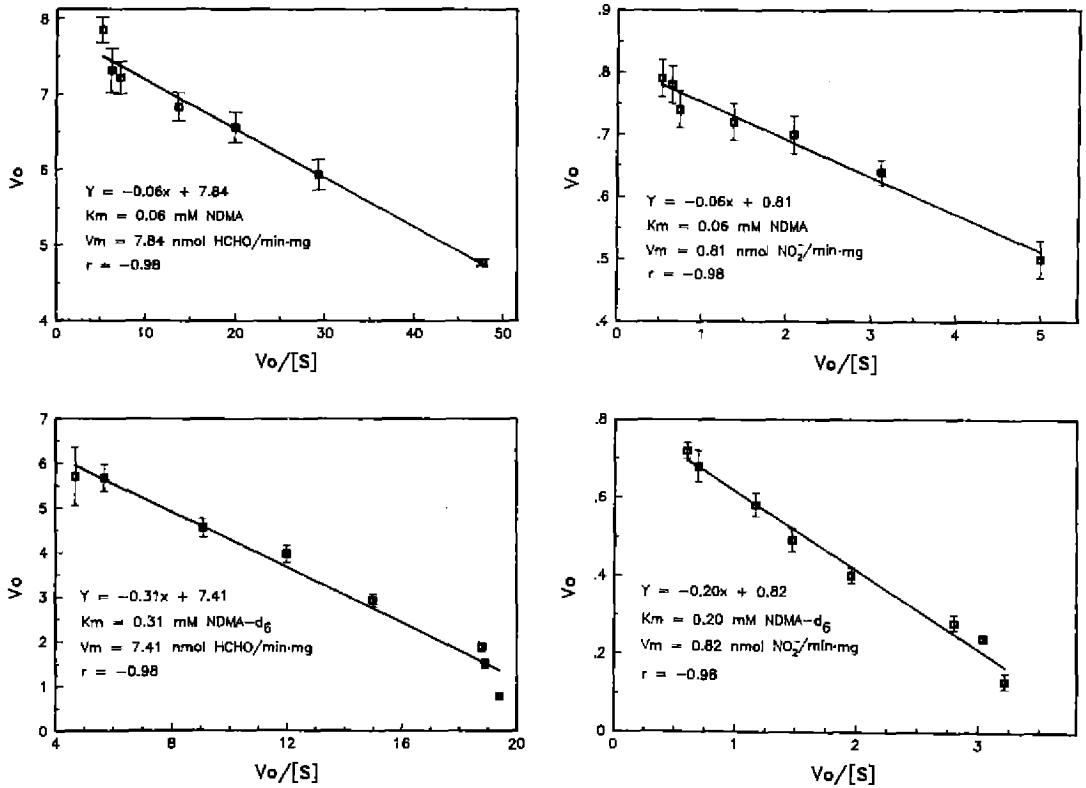
As a first approach, the means of the data points from different experiments were plotted (Fig. 1). Apparent  $K_m$  values of 0.06 mM were observed for both the demethylation and denitrosation reactions, whereas apparent  $V_{max}$  values of 7.8 and 0.81 nmol/min per mg, respectively, were observed for these two reactions. With deuterated substrate, the  $V_{max}$  was not significantly altered, but the  $K_m$  value was increased three to five fold. A similar conclusion was reached with the  $K_m$  and  $V_{max}$  obtained from Lineweaver-Burk plots.

In a second approach, the data from each set of experiments were graphed as Eadie-Hofstee plots; the different kinetic parameters are summarized in Table 1. The results were essentially the same as those from the first approach.

#### Kinetic isotope effect

The data of Table 1 may be used to calculate the values of the observed isotope effects on  $V_{max}$ ,  $K_m$  and the ratio of  $V_{max}:K_m$ , for both the demethylation and denitrosation reactions. The ratios of  $V_{max}:V_{max}^D$  are equal to one for both the demethylation and denitrosation reactions, which indicates that there was no isotope effect on  $V_{max}$  in either reaction (Northrop, 1982). This result implies that either the overall rates of demethylation and denitrosation under saturating conditions are not controlled by a slow catalytic step involving the cleavage of a carbon-hydrogen bond or the cleavage step is only one of several rate-limiting steps.

**Fig. 1. Eadie-Hofstee plots of microsomal demethylation and denitrosation of NDMA and NDMA-d<sub>6</sub>**



The reaction mixture (1 ml) contained acetone-induced rat-liver microsomes (0.8 mg protein) and 0.04, 0.08, 0.10, 0.20, 0.33, 0.50, 1.00 or 1.50 mM NDMA or NDMA-d<sub>6</sub>. The means and standard errors from nine sets of experiments are shown. Upper left, demethylation of NDMA; upper right, demethylation of NDMA-d<sub>6</sub>; lower left, denitrosation of NDMA; lower right, denitrosation of NDMA-d<sub>6</sub>.

**Table 1. Kinetic parameters of microsomal demethylation and denitrosation<sup>a</sup>**

	$K_m$	$V_{max}$ (nmol/min-mg)
Demethylation		
NDMA	$0.06 \pm 0.01$	$7.92 \pm 0.67$
NDMA-d <sub>6</sub>	$0.30 \pm 0.03^b$	$7.73 \pm 1.44$
Denitrosation		
NDMA	$0.06 \pm 0.02$	$0.83 \pm 0.06$
NDMA-d <sub>6</sub>	$0.19 \pm 0.06^b$	$0.83 \pm 0.15$

<sup>a</sup>The data, expressed as mean  $\pm$  SD, are from nine experiments, except for the demethylation of NDMA, which was from 14 experiments.

<sup>b</sup>Significantly different from  $K_m$  for NDMA ( $p < 0.001$ )

With regard to the ratio of  $K_m^H:K_m^D$ , the calculated values for the demethylation and denitrosation reactions were 0.2 and 0.3, respectively. These results indicate that there were isotope effects on the  $K_m$  in both reactions; however, the interpretation of such effects may be obscured by the fact that the  $K_m$  is a complex constant. If the  $K_m$  is mainly a reflection of the dissociation constant of the enzyme-substrate complex, then an isotope

effect on  $K_m$  can be used as a measure of the relative affinity of the substrate for the enzyme. This condition is satisfied when the rate constant for conversion of enzyme-substrate complex to product is negligible in comparison with the rate constant for dissociation of the complex in the rate equation (Piszkiewicz, 1977). Under these conditions, the isotope effects on  $K_m$  may be interpreted as indicating that the deuterated form of NDMA is bound less strongly to cytochrome P450 than is the undeuterated form.

The ratio of  $V_{max}:K_m$  is, like  $K_m$ , a complex function of several rate constants (Walsh, 1979). In spite of this complexity, it is possible to examine isotope effects on this ratio to obtain a better understanding of the events involved in an enzyme-catalysed reaction. The ratio can be related to the rates of formation, and of dissociation, of the enzyme-substrate complex. An apparent isotope effect on this ratio is suppressed when the rate of conversion of enzyme-substrate complex to enzyme-product complex is much greater than the rate of dissociation of enzyme-substrate complex into enzyme and substrate. However, the effect is fully expressed in the converse situation, i.e., when the rate of dissociation of enzyme-substrate complex greatly exceeds the rate of conversion to enzyme-product complex. Calculations of  $(V_{max}:K_m)^H/(V_{max}:K_m)^D$  resulted in values of 5.1 and 3.2 for demethylation and denitrosation, respectively. These results indicate that isotope effects on  $V_{max}:K_m$  were expressed in both of the reactions considered. When an isotope effect is expressed in the  $V_{max}:K_m$  ratio, but not in  $V_{max}$  (as is the case in this experiment), this indicates that isotopic substitution has revealed a low commitment to catalysis. In other words, the dissociation of substrate from the enzyme-substrate complex occurs much more readily than covalent change to the enzyme-product form. The results of these experiments are not unique. Studies with other enzymes such as alcohol dehydrogenase have shown that isotopic substitution results in effects on  $V_{max}:K_m$ , but not on  $V_{max}$  (Walsh, 1979).

### Discussion

The similarity in  $K_m$  values for denitrosation and demethylation of NDMA supports previous suggestions (Lorr *et al.*, 1982; Tu & Yang, 1985) that these two reactions are closely related and may share a common initial step. However, the nature of the data on denitrosation does not allow us to conclude whether the deuteration of NDMA affects the two types of reactions to the same or to a different extent.

The results with regard to demethylation are different from those of Dagani and Archer (1976), who found that deuteration caused a reduction in the  $V_{max}$  and  $K_m$  for demethylation of high concentrations of NDMA by liver microsomes from phenobarbital-induced rats. The decrease in  $K_m$  was interpreted as being due to the greater affinity of the enzyme for fully deuterated NDMA- $d_6$  than for its undeuterated analogue. Using much lower substrate concentrations and the 9000  $\times$  g supernatant fraction from the livers of a different strain of rat, Kroeger-Koepke and Michejda (1979) also observed deuterium isotope effects on the  $V_{max}$  and  $K_m$  for demethylation of NDMA. They suggested that the results might indicate that cleavage of the carbon-hydrogen bond was rate-determining in the enzymatic oxidation of NDMA. The origin of the difference between the findings of those authors and ours is not known, but it may be due to the fact that those authors studied the NDMA demethylase activities of cytochrome P450 isozymes that are different from the low  $K_m$  isozymes (Yang *et al.*, this volume) studied here. By using acetone-induced microsomes and a substrate concentration ranging from 0.04-1.5 mM, we believe that we

have studied the low- $K_m$  (0.06 mM) form of NDMA demethylase, the form of enzyme activity that is believed to be important in the metabolism of NDMA *in vivo* (Heath, 1962; Tu & Yang, 1983). The observed increase in  $K_m$  value, due to the deuteration of NDMA, provides a basis for interpreting previous observations (Swann *et al.*, 1983; Mico *et al.*, 1985), which demonstrated a large isotope effect when NDMA- $d_6$  and NDMA were administered to rats as a mixture, but a small isotope effect when they were administered separately.

## STRUCTURE-ACTIVITY RELATIONSHIPS IN METABOLISM AND MUTAGENICITIES OF *N*-NITROSAMINES

J.B. Guttenplan

*Department of Environmental Medicine, New York University Medical School  
and Department of Biochemistry, New York University Dental School,  
New York, NY, 10010, USA*

The metabolism of a series of nitrosamines *in vitro* was monitored by measuring nitrogen production and was compared with mutagenesis by the same compounds, allowing separation of mutagenic potencies into metabolic and postmetabolic terms. The rate of nitrogen production from symmetrical di-*n*-alkyl and methylalkyl nitrosamines increased with increasing molecular weight. The cyclic nitrosamines *N*-nitrosopiperidine and *N*-nitrosopyrrolidine were metabolized slightly less rapidly than the most hydrophobic compounds, and *N*-nitrosomorpholine was metabolized at about half this rate. *N*-Nitrosomethylaniline was metabolized to nitrogen relatively slowly. Branching at the  $\alpha$ -carbons reduced  $\alpha$ -oxidative metabolism several fold. Substitution at the  $\beta$ -carbon of *N*-nitrosodiethylamine or *N*-nitrosodi-*n*-propylamine with hydroxyl, cyano, oxo and methoxyl groups reduced metabolism to an even greater extent. Carboxyl substitution at the 4-position of *N*-nitrosopiperidine greatly reduced nitrogen formation, but 4-*tert*-butyl substitution had little effect. Effects of structure on mutagenic activities in *Salmonella* followed a different pattern. Higher homologue di-*n*-alkyl nitrosamines were more potent than lower homologues at lower doses, when potencies were taken from slopes of dose-response curves. However, when mutagenic potencies were expressed as 'mutagenic efficiencies' (revertants/ $\mu$ mol nitrogen), regardless of dose, the order of potency was *N*-nitrosodimethylamine > *N*-nitrosodiethylamine > *N*-nitrosodi-*n*-propylamine > *N*-nitrosodibutylamine. For the series of methylalkyl nitrosamines, mutagenic potencies were greatest for the higher molecular weight compounds, but they were all similar to that of *N*-nitrosodimethylamine when expressed as mutagenic efficiencies. Branching and the presence of functional groups reduced mutagenesis, sometimes to a greater extent than nitrogen production, indicating that factors other than reduced metabolism lead to reduced mutagenesis by these groups. A reasonable correlation between mutagenic efficiencies and known carcinogenic potencies for these compounds was observed.

*N*-Nitrosamines represent a class of compounds containing a high percentage of carcinogens and mutagens of greatly diverse potencies. In order to investigate the basis for some of their different mutagenic effects, mutagenesis induced by nitrosamines in *Salmonella typhimurium* was compared with the  $\alpha$ -oxidative metabolism (monitored by quantification of the product, nitrogen) of a series of nitrosamines, and differences in mutagenic potencies were separated into metabolic and post-metabolic terms. Although many studies of comparative mutagenesis by different nitrosamines have been reported, few, if any, monitored metabolism and mutagenesis under the same conditions. Thus, the reasons given for the different mutagenic activities are largely speculative.

### Mutagenesis

The mutagenic activities of a series of acyclic nitrosamines are given in Table 1. Mutagenicities in *Salmonella typhimurium* TA100 were calculated from slopes of linear portions of dose-response curves. Compounds branched at both  $\alpha$ -carbons (*N*-nitrosodiisopropylamine and *N*-nitrosodisecbutylamine) were much weaker than their unbranched analogues, and compounds containing hydroxyl, keto, cyano and methoxyl substituents were much weaker than their unsubstituted analogues. *N*-Nitrosomethylaniline was also a weak mutagen in TA100 but was much more potent in TA104. This compound and *N*-nitrosodiethylamine were the only nitrosamines to exhibit this effect. The mutagenesis value for *N*-nitrosomethylaniline in Table 1 is given for TA104. Among the cyclic nitrosamines, *N*-nitrosopiperidine and *N*-nitrosomorpholine were the most potent, and the substituents  $\alpha$ -dimethyl, 4-carboxy, 4-hydroxy and 4-keto all reduced mutagenesis by *N*-nitrosopiperidine. *N*-Nitrosopyrrolidine was less potent than *N*-nitrosopiperidine, and chloro substitution greatly increased the mutagenesis of *N*-nitrosopyrrolidine.

### Nitrogen production

Metabolism of unbranched nitrosamines to nitrogen reflected the hydrophobicity of the nitrosamines, the more hydrophobic nitrosamines generally being metabolized most rapidly to nitrogen. *N*-Nitrosomethylaniline was an exception and was metabolized to nitrogen several times less rapidly than the other methyl nitrosamines. Branching at the  $\alpha$ -carbon and the incorporation of polar substituents (hydroxyl, keto, carboxy, cyano, methoxyl) reduced nitrogen generation many fold. Substitution of chlorines in *N*-nitrosopyrrolidine reduced nitrogen production by about two fold. Nitrogen generation from *N*-nitrosomorpholine was several fold less than that from *N*-nitrosopiperidine and *N*-nitrosopyrrolidine.

### Comparison of mutagenic activities and nitrogen generation

Mutagenicity values were expressed relative to nitrogen production (both determined at the same concentration) to yield 'mutagenic efficiencies' of activated nitrosamines. Expression of potency data in this form normalizes all mutagenicity data to the same amount of metabolism of nitrogen. Factors that led to decreased metabolism (substitution with hydroxyl, cyano, carboxyl and methoxyls and  $\alpha$ -branching) often led to decreased mutagenesis, but the decrease in mutagenesis sometimes tended to be larger than that for metabolism, indicating that post-activational processes (e.g., cellular permeation, site and extent of DNA adduct formation and DNA repair) also lead to reduced mutagenic activities. In contrast, the differences in the mutagenic activities of the methylalkyl nitrosamines were largely ascribable to different rates of metabolism, as was the difference in mutagenesis between *N*-nitrosopiperidine and *N*-nitroso-4-piperidone, as seen from the similar mutagenic efficiencies of these compounds.

Also shown in Table 1 are the carcinogenic potencies for the nitrosamines in rats (Lijinsky, 1984b). There is a reasonable correlation between mutagenic efficiencies and carcinogenic potencies, whereas there is no potency correlation if mutagenesis is expressed simply as the slope of the dose-response curve. As most nitrosamines are metabolized to the same extent (completely) *in vivo*, but not *in vitro*, expression of nitrosamine mutagenicity data, normalized to the same amount of metabolism to nitrogen, may be more relevant to carcinogenic potency than the slopes of dose-response curves.

**Table 1. Relationships between nitrogen generation from, mutagenesis by, and carcinogenesis by *N*-nitrosamines: acyclic and cyclic nitrosamines—cyclic and acyclic nitrosamines**

<i>N</i> -Nitrosamine (2 mg/ml)	Nitrogen generated <sup>a</sup>	Mutagenesis <sup>b</sup> (rev/10 <sup>8</sup> per μmol nitros- amine)	Mutagenic efficiency (rev/μmol nitrogen) <sup>c</sup>	Carcinogenic potency <sup>d</sup>
<i>Acyclic nitrosamines</i>				
butylmethyl	1.55	1060	40 600	++++
ethylmethyl	0.85	575	25 411	+++
dimethyl	0.58	270	18 100	++++
diethyl	1.03	300	24 300	++++
dipropyl	0.98	1307	5 300	+++
phenylmethyl <sup>e</sup>	0.24	210	3 017	+++
bis(2-methoxyethyl)	0.21	35	2 190	+++
dibutyl	0.96	1075	1 250	++
bis(2-hydroxyethyl)	0.13	3.1	376	+
diisopropyl	0.23	5.0	348	+
bis-2-(cyanoethyl)	0.15	2.6	246	0
di- <i>sec</i> -butyl	0.21	0	0	0
<i>Cyclic nitrosamines</i>				
morpholine	0.24	1480	21 040	+++
3,4-dichloropyrrolidine	0.58	2680	8 482	++++
piperidine	1.04	1560	6 380	+++
4-piperidone	0.18	40	3 611	+++
4-hydroxypiperidine	0.27	44	2 629	+++
pyrrolidine	0.93	450	1 086	++
4- <i>tert</i> -butylpiperidine	0.76	570	868	+
2,6-dimethylpiperidine	0.19	5.7	63	0
4-carboxypiperidine	0.23	0	0	0

<sup>a</sup>Nitrogen was assayed by carrying out in-vitro metabolism in septum-sealed (vacutainer) blood collection tubes containing S9 fraction, NADPH and a magnetic stirbar, and were capped. The tubes were purged for 30 min with oxygen *via* inlet and exit port needles, and nitrosamine was then added with a syringe. The mixtures were incubated for 90 min, and a sample of the headspace was analysed for nitrogen by gas chromatography. Liver S9 fractions from Aroclor 1254-induced rats were used in all experiments.

<sup>b</sup>Taken from the slope of the linear portion of dose-response curves. Mutagenesis in *S. typhimurium* TA100 was determined as described previously (Guttenplan *et al.*, 1980; Lee & Guttenplan, 1981).

<sup>c</sup>Taken from mutagenicity value at 2 or 0.2 mg/ml nitrosamine and divided by the nitrogen value determined at the same concentration of nitrosamine

<sup>d</sup>From Lijinsky (1984)

<sup>e</sup>Mutagenesis assayed in TA104

### Acknowledgements

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## THE PIG AS AN ANIMAL MODEL FOR THE STUDY OF NITROSAMINE METABOLISM

G.W. Harrington<sup>1</sup>, P.N. Magee<sup>2</sup>, H.M. Pylypiw, Jr<sup>1</sup>, R.F. Bevil<sup>2</sup>,  
D.R. Nelson<sup>3</sup> & J.C. Thurmon<sup>3</sup>

<sup>1</sup>Department of Chemistry, Temple University, Philadelphia, PA 19122, USA; <sup>2</sup>Fels Research Institute and Department of Pathology, Temple University, School of Medicine, Philadelphia, PA 19140, USA; and <sup>3</sup>College of Veterinary Medicine, University of Illinois, Urbana, IL 61801, USA

**Surgical procedures have been developed that permit the sampling of portal blood, bile and hepatic blood in intact pigs. Animals so prepared have been used to study liver metabolism and biliary excretion of *N*-nitrosodimethylamine (NDMA) following oral and intravenous dosing.**

Although the metabolism of *N*-nitrosamines has been investigated extensively, most metabolic studies have been conducted *in vitro* or in small laboratory animals such as the rat (e.g., Magee, 1956; Mico *et al.*, 1985). The pig, by virtue of its size, adaptability to confinement and the remarkable similarity of its gastrointestinal physiology to that of humans (Bustad, 1966), is the ideal animal for metabolic studies. Its size permits repeated collection over long periods of time of relatively large samples of biological fluids and, after sacrifice, the collection of enormous samples of tissues and organs for nitrosamine analyses and for the preparation of nucleic acids and proteins for alkylation studies. In addition, several experiments can be carried out on a single animal, and each animal can serve as its own control.

Oral or intravenous dosing is quite simple and, in addition, the pig is an omnivorous animal that will eat human food and thus can be fed diets reported to give rise to detectable nitrosamines in humans.

Thus, procedures have been developed for cannulation of the portal and hepatic veins as well as the gallbladder for collection of bile. The animals undergo rapid recovery from surgery and thus provide an excellent living model for metabolic studies.

### **Surgical procedures**

The animals used were selected from a specific pathogen-free herd maintained at the College of Veterinary Medicine, University of Illinois, USA. This is a genetically controlled herd of Chester White X Duroc pigs.

Anaesthesia was induced and maintained with halothane in oxygen. A laparotomy was performed, and a catheter (Rothene tubing, Electro Catheter Corp., Rahway, NJ, USA) introduced into the portal vein. The position of the catheter tip was determined by palpation. Another catheter was introduced into the posterior vena cava and advanced to the site of entry of the hepatic vein. The final position of the catheter tip was determined by palpation. The procedure of Dougherty *et al.* (1965) was used for implantation of a catheter into the femoral artery or vein.



A catheter was introduced into the free end of the gallbladder for bile collection. An occluder cuff (In Vivo Metric Systems, Healdsburg, CA, USA) was placed around the bile duct proximal to its entrance into the duodenum. Occlusion of the bile duct prevented bile flow into the duodenum and allowed its collection from the gallbladder. Release of the occluder cuff permitted normal bile flow to return to the duodenum.

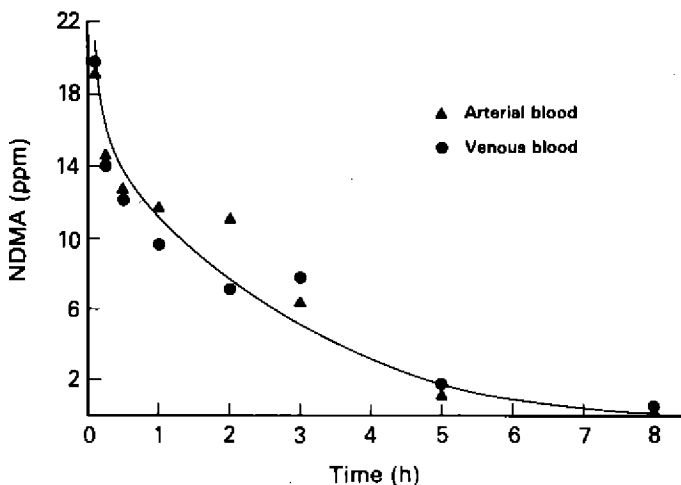
Following surgery, the animals were placed in metabolic cages and allowed to recover for 24 h prior to dosing and sampling. Oral dosing was performed using a stomach tube and a mouth speculum. Animals dosed orally were fasted for 24 h prior to dosing.

Samples were frozen immediately upon collection. Analysis for NDMA was by gas chromatography-thermal energy analysis using the procedure of Pylypiw *et al.* (1985).

## Results

The disappearance and distribution of NDMA in the pig is very similar to that observed in the rat (Mico *et al.*, 1985), as shown by the data in Figure 1. Ten mg/kg NDMA were injected intravenously, and arterial and venous blood samples were collected at selected times and analysed for NDMA. The figure shows that the decay curves are virtually superimposable and suggests that the NDMA rapidly becomes equilibrated throughout the body. Lower doses show proportionally faster decay times.

Fig. 1. Disappearance of NDMA from peripheral blood

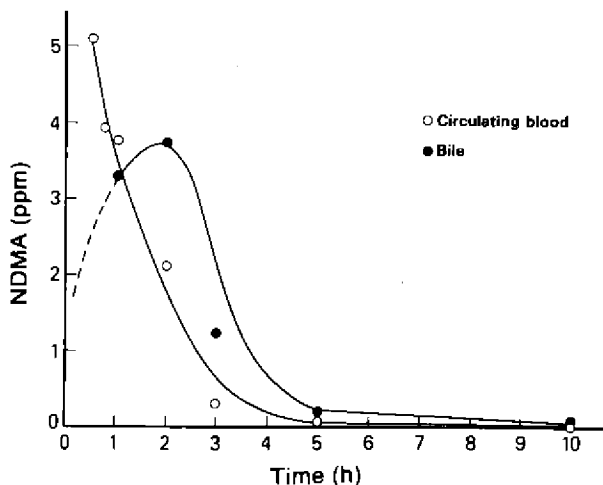


Initial dose: 10 mg/kg body weight

In other experiments, animals were dosed *via* interarterial, intravenous and oral routes. Peripheral blood, bile and portal and hepatic blood were collected at selected time intervals. The dosing levels were 10 mg, 5 mg, 1.0 mg and 0.1 mg/kg body weight. Figure 2 shows the relationship between NDMA levels in peripheral blood and bile following interarterial dosing at 10 mg/kg body weight. It can be seen that the level in bile reaches that in peripheral blood after 1 h, and then both levels decay in parallel. NDMA levels in bile always lag behind blood levels, whether peripheral blood or portal or hepatic blood.

Figure 3 shows NDMA levels in portal and hepatic blood following oral dosing at three different levels. The data points in the graphs represent the means from two animals at the 10-mg dose, four animals at the 1-mg dose and two animals at the 0.1-mg dose. Scatter bars are not shown since they would be too small to be drawn effectively: reproducibility from one animal to another is remarkably good.

**Fig. 2. Disappearance of NDMA from peripheral blood and bile**



Dose: 10 mg/kg body weight intraarterially

It is interesting to note that even at the relatively high dose of 10 mg/kg, which corresponds to 700 mg of the compound given to the average-sized human, very little NDMA could be detected after 2 h, and that, when 0.1 mg/kg was given, corresponding to a dose of 7.0 mg to a human, NDMA was virtually undetectable after 1.5 h. The peaks seen with the lower dose are probably due to absorption from the stomach followed by absorption from the small intestine.

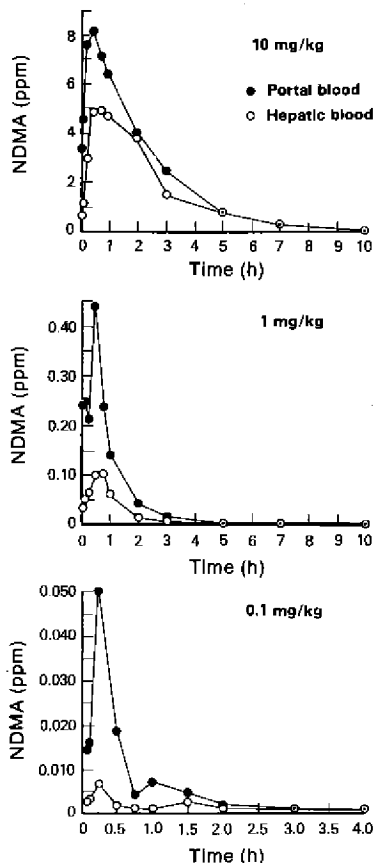
The effective removal of NDMA from blood passing through the liver is clearly demonstrated in these studies, nearly complete removal occurring after an oral dose of 0.1 mg/kg. These results are consistent with the earlier evidence for a first-pass mechanism for the removal of NDMA by rat liver.

This model should be extremely useful for metabolic and other types of studies involving *N*-nitrosamines and related compounds and should provide information more directly related to the human than is possible using the usual laboratory animals.

#### Acknowledgements

This work was supported by grants No. CA-18618 and CA 23451 from the National Institutes of Health, USA, by Grant SIG-6 from the American Cancer Society and by grants from the National Foundation for Cancer Research and from the Samuel S. Fels Fund of Philadelphia.

**Fig. 3. NDMA levels in portal versus hepatic blood at three oral dosing levels**



## THE FERRET AS A MODEL FOR ENDOGENOUS SYNTHESIS AND METABOLISM OF *N*-NITROSAMINES

J.S. Wishnok<sup>1,3</sup>, S.R. Tannenbaum<sup>1</sup>, J.G. Fox<sup>2</sup>, J.E. Mesina<sup>2</sup> & L.C. Hotaling<sup>2</sup>

<sup>1</sup>*Department of Applied Biological Sciences and*

<sup>2</sup>*Department of Comparative Medicine, Massachusetts Institute of Technology, Cambridge, MA 02139, USA*

We have begun to evaluate the ferret as a model for studying gastric nitrosation and nitrosamine metabolism. Jugular cannulation allows convenient dosing and sampling of blood, and this technique has been used to study clearance of *N*-nitrosodimethylamine (NDMA) after intravenous, intraperitoneal and intragastric administration. NDMA metabolism can be inhibited by 4-methylpyrazole for up to 24 h; the accumulation of endogenously-synthesized NDMA can therefore be observed in animals following pre-treatment with this compound. We have studied endogenous NDMA synthesis in animals dosed with dimethylamine and nitrite and in animals to which no NDMA precursors were administered.

Endogenous (especially gastric) synthesis of nitrosamines has been studied for over 15 years, but the inadequacies of many animal models were noted at least as early as 1969, when Sen and co-workers suggested that ferrets, among other animals, might prove better models for human gastric nitrosation than, for example, rats. A major goal in our laboratories has been to develop methods that will allow extrapolation from animals to humans. The ferret is intermediate in size between the rat and the dog, and its stomach is anatomically and physiologically similar to the human stomach. Additionally, the activities of drug-metabolizing enzymes in ferrets are similar to those in rats and humans (Hoar, 1984). The animal is large enough to allow essentially harmless removal of up to 10 ml of blood *via* semi-permanent catheters, and small enough that costs of purchase and upkeep are not prohibitive. We have consequently begun an evaluation of the ferret as a model for gastric nitrosation and for studying the detailed metabolic fate of *N*-nitroso compounds.

### Experimental methods

*Jugular catheters:* Ferrets were anaesthetized with ketamine and xylazine and the jugular vein exposed and loosely looped with two sutures. A polyethylene tube was inserted into a 19-gauge needle and the needle pushed into the vein past the suture loops. The needle was withdrawn, leaving the catheter, which was secured by tightening and tying the sutures. A second incision was then made behind the animal's ear and a 17-gauge needle inserted and pushed subcutaneously to the site of the jugular incision. The free end of the catheter was inserted into this needle and threaded through. The needle was gently removed and the incisions closed. For clearance and metabolism experiments, the end of the catheter was fitted with a three-way stopcock for convenient dosing, sampling and flushing of the catheter. Animals were allowed to recover from surgery for at least one week before additional experiments.

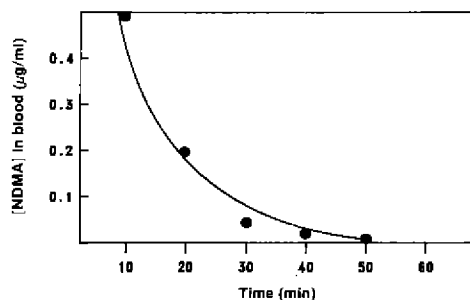
**Metabolism of NDMA:** In a typical experiment, NDMA was dissolved in phosphate-buffered saline and administered intravenously *via* the jugular catheter, intragastrically by gavage or intraperitoneally. Samples (0.2 ml) of blood were withdrawn through the catheter and the volume was replaced with 0.2 ml phosphate-buffered saline. The blood was added to centrifuge tubes containing 0.2 ml each distilled water (containing *N*-nitrosodiethylamine as internal standard) and methylene chloride, and the tubes were closed and shaken vigorously. Just prior to analysis, the tubes were agitated a second time in a vortex mixer and then centrifuged for several minutes to separate the layers. The methylene chloride layer was then analysed directly by gas-chromatography-thermal energy analysis.

**Endogenous NDMA formation:** Catheterized ferrets were allowed to recover from surgery and were dosed intraperitoneally with 4-methylpyrazole, to slow the metabolism of any synthesized NDMA, at least 1 h prior to administration of any precursors. Dimethylamine and sodium nitrite were administered in phosphate-buffered saline. The blood was sampled and analysed as described above.

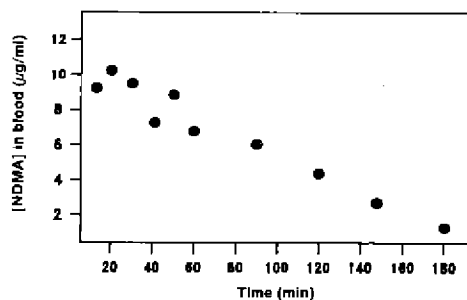
### Results and discussion

The rate of clearance of NDMA from ferret blood is comparable to that of rats; an intravenous dose of 1 mg/kg is typically metabolized within 1 h; the best fit for the data are first-order curves giving half-lives of 6-19 min (Fig. 1). A dose of 20 mg/kg produced easily detectable concentrations of NDMA for as long as 3 h and disappeared *via* zero-order kinetics during most of this time (Fig. 2). The animal used in this experiment died with acute liver toxicity. The maximum concentration of NDMA is reached about 20 min following intraperitoneal administration and about 30 min after intragastric administration.

**Fig. 1. Metabolism of NDMA (1 mg/kg bw) administered intravenously *via* a jugular catheter**

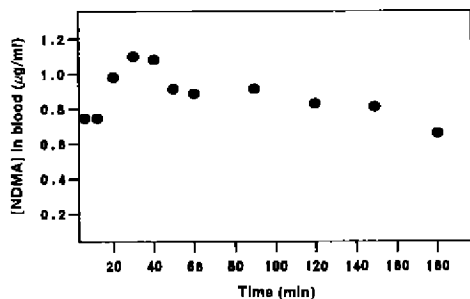


**Fig. 2. Metabolism of NDMA (20 mg/kg bw) administered intraperitoneally**



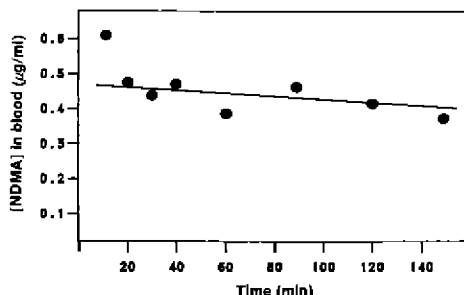
The metabolism of NDMA can be inhibited with 4-methylpyrazole (50-100 mg/kg intraperitoneally 1 h prior to NDMA administration; Phillips *et al.*, 1974; Skipper *et al.*, 1983); substantial inhibition is observed for at least 2.5 h (Figs 3 and 4), and some effects persist for up to 24 h. Typical clearance rates are observed within four days of an inhibition experiment, i.e., the 4-methylpyrazole does not appear to have long-term effects. Inhibition of NDMA metabolism also allows the observation of endogenous formation of this compound: Figure 5 shows the appearance of NDMA in blood following intragastric administration of dimethylamine and nitrite to a 4-methylpyrazole-treated ferret.

**Fig. 3. Inhibition of NDMA metabolism by 4-methylpyrazole**



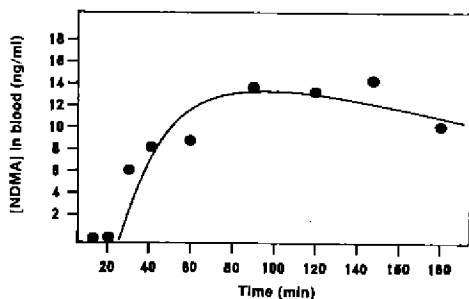
NDMA (1 mg/kg bw) administered intragastrically 1 h after administration of 4-methylpyrazole

**Fig. 4. Inhibition of NDMA metabolism by 4-methylpyrazole**



NDMA (1 mg/kg bw) administered intravenously via a jugular catheter 1 h after administration of 4-methylpyrazole

**Fig. 5. Endogenous NDMA synthesis in a ferret pretreated with 4-methylpyrazole; dimethylamine (10 mg/kg) and nitrite (15 mg/kg) by gavage**



We have shown that the ferret can readily be used to study NDMA metabolism and endogenous synthesis. Previous workers have shown that ferrets can biosynthesize nitrate (Dull & Hotchkiss, 1984a) and that a constant level of dimethylamine of unknown origin is present in ferret stomach (Zeisel *et al.*, 1985). This animal is therefore, from several view points (i.e., experimental convenience, economy, biology and physiology), an excellent model for more extensive studies in these areas.

**Acknowledgements**

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## CELL TYPE-SPECIFIC DIFFERENCES IN METABOLIC ACTIVATION OF *N*-NITROSODIETHYLAMINE BY HUMAN LUNG CANCER CELL LINES

H.M. Schuller<sup>1</sup>, M. Falzon<sup>2</sup>, A.F. Gazdar<sup>3</sup> & T. Hegedus<sup>1</sup>

<sup>1</sup>Laboratory of Experimental Oncology, Department of Pathobiology,  
College of Veterinary Medicine, University of Tennessee, Knoxville, TN, USA;

<sup>2</sup>Laboratory of Experimental Carcinogenesis, DCE, National Cancer Institute, Bethesda,  
MD, USA; and <sup>3</sup>NCI-Navy, Medical Oncology Branch, Naval Hospital,  
Bethesda, MD, USA

The metabolism of *N*-nitrosodiethylamine (NDEA) and its modulation by inhibitors of cytochrome P450 and prostaglandin H synthetase enzymes was investigated in seven well-differentiated early-passage human lung cancer cell lines. NDEA metabolism was assessed by covalent binding and evolution of carbon dioxide. Morphological diagnosis of cell lines was done by light and electron microscopy. Two cell lines (NCI-H69, NCI-H128) with characteristics of small-cell cancer did not metabolize NDEA. Two cell lines (NCI-H322) with features of adenocarcinoma, comprised of Clara cells, and (NCI-H727), with features of pulmonary endocrine cells, were more potent than all other cell lines in metabolizing NDEA. A cell line divided from an adenocarcinoma but comprised of alveolar type-II cells (NCI-H358) metabolized NDEA predominantly *via* prostaglandin H synthetase. Similarly, several cell lines with features of well-differentiated pulmonary endocrine cells (NCI-H727, NCI-H460) metabolized NDEA *via* prostaglandin H synthetase, while the cell line comprised of Clara cells (NCI-H322) activated the nitrosamine by cytochrome P450 but not by prostaglandin H synthetase. Although cancer cells may react differently from normal cells to xenobiotics, our data provide substantial evidence for the hypothesis that — as in the hamster — Clara cells and pulmonary endocrine cells are potential major targets of NDEA carcinogenesis in human lung. It is of particular interest that different cell types activate the nitrosamine *via* different enzyme systems.

NDEA is a potent respiratory-tract carcinogen in hamsters. The induced pulmonary adenocarcinomas are derived from Clara cells, which show selective covalent binding after *in-vivo* administration of <sup>14</sup>C-NDEA (Reznik-Schuller & Reznik, 1979) along with selective formation of *O*<sup>6</sup>-ethylguanine (Fong & Rasmussen, 1985). To assess the relevance of such data for human carcinogenesis, NDEA metabolism by human lung cancer cell lines was studied. Well-differentiated human lung cancer cell lines at early passages are excellent model systems to study cell type-specific pathways of metabolic activation. They retain most of the morphological and biochemical characteristics of their normal cells of origin and are comprised of a homogeneous population of one cell type.

### Morphology of cell lines

The cell lines were characterized morphologically by transmission electron microscopy (glutaraldehyde, osmium fixation, embedded in Epon, uranyl acetate and lead citrate stain). Two cell lines (NCI-H69 and NCI-H128) were diagnosed as small-cell cancer lines. Cell line NCI-H322 (passage 15) showed features of adenocarcinomas comprised of Clara cells, while NCI-H358 (passage 15) demonstrated features of adenocarcinoma comprised of alveolar type-II cells. Lines NCI-H727 (passage 8) and NCI-H460 (passage 11) showed features of well-differentiated pulmonary endocrine cells.

### Metabolism of NDEA

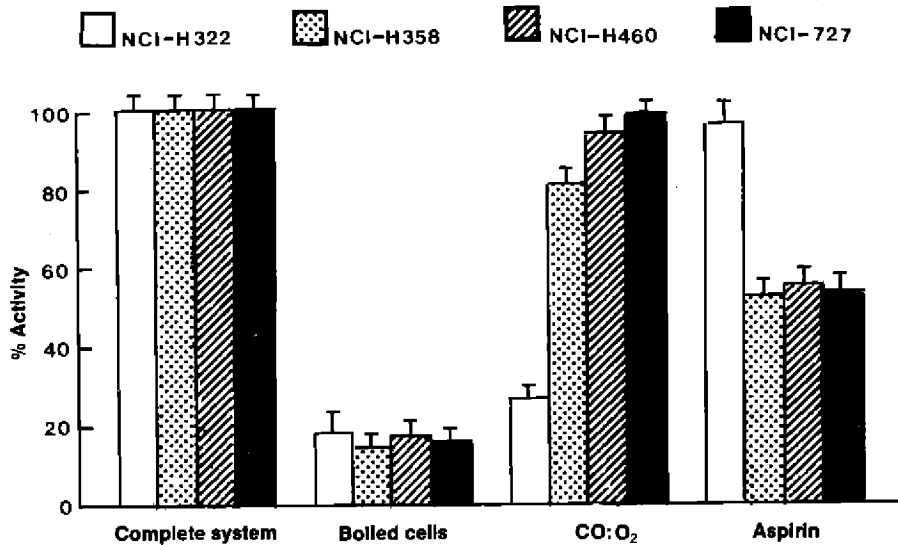
Metabolism of NDEA was assessed by evolution of carbon dioxide and covalent binding to DNA after incubation for 10 min with  $^{14}\text{C}$ -NDEA at a final concentration of 1 mM. The effect of the cytochrome P450 inhibitor carbon monoxide was assessed by bubbling a carbon monoxide-oxygen mixture through the cells suspended in culture medium for 10 min before incubation with  $^{14}\text{C}$ -NDEA. As shown in Table 1, the cell lines with Clara cell morphology (NCI-H322) and endocrine cell morphology (NCI-H727) were the most potent of the investigated cell lines in metabolizing NDEA. Preincubation of the cell lines with the various enzyme inhibitors (Fig. 1) almost completely inhibited NDEA metabolism in line NCI-H322 (Clara cell morphology) with the carbon monoxide-oxygen mixture (a condition known to inhibit cytochrome P450). In contrast, this condition did not result in any significant reduction of NDEA metabolism in the two cell lines with features of pulmonary endocrine cells (NCI-H727, NCI-H460). Aspirin and indomethacin, which are known inhibitors of the fatty acid cyclooxygenase component of prostaglandin endoperoxide synthetase, reduced NDEA metabolism in these two cell lines by 50% (Fig. 1). The cell line with features of alveolar type-II cells (NCI-H358) appeared to metabolize NDEA preferentially *via* prostaglandin endoperoxide synthetase, while cytochrome P450 was involved only marginally (Fig. 1). Our data on the involvement of prostaglandin H synthetase in NDEA metabolism by cell lines NCI-H358, NCI-H727 and NCI-H460 are supported by the detection of high levels of prostaglandin  $\text{E}_2$  in these cell lines after incubation with arachidonic acid (Lau *et al.*, 1986; Schuller, unpublished data).

**Table 1. Metabolism of  $^{14}\text{C}$ -NDEA by human lung cancer cell lines**

Cell line	Morphology	$^{14}\text{C}$ -carbon dioxide (pmol/10 min per mg protein)
NCI-H128	Small-cell cancer (poorly differentiated)	Not detectable
NCI-H69	Small-cell cancer (poorly differentiated)	Not detectable
NCI-H322	Adenocarcinoma: Clara cell morphology	829.9 $\pm$ 21.1
NCI-H358	Adenocarcinoma: alveolar type-II cell morphology	466.6 $\pm$ 18.4
NCI-H460	Large-cell carcinoma: endocrine cell morphology (well-differentiated)	3690 $\pm$ 22
NCI-H727	Carcinoid: endocrine cell morphology (well differentiated)	28925 $\pm$ 56.7

Cells were incubated with 1 mM  $^{14}\text{C}$ -NDEA concentration in tissue culture medium for 10 min. Values are means and standard deviations of triplicate determinations.

**Fig. 1. Evolution of  $^{14}\text{C}$ -carbon dioxide following incubation with  $^{14}\text{C}$ -NDEA under various conditions**



Although we realize that cancer cells may react differently to xenobiotics and to normal cells, we feel that our data provide substantial evidence for the hypothesis that — as in the hamster — NDEA has a pronounced specificity for Clara cells and pulmonary endocrine cells in human lung. This is of particular interest inasmuch as these two cell types are the cells of origin of peripheral adenocarcinoma and small-cell carcinoma, respectively; these are the types of lung cancer most commonly linked with cigarette smoking in recent years (Wynder *et al.*, 1985). In conjunction with the data from our animal experiments (Reznik-Schuller & Reznik, 1979), the present findings support the assumption that nitrosamines play a much more important role in tobacco-linked lung carcinogenesis than previously suspected. Our data also show that efforts to prevent the induction of lung cancer by tobacco-derived and other nitrosamines by blocking their metabolic activation will face considerable problems since such activation depends on different enzyme systems in different cell types.



# A COMPARATIVE STUDY OF THE MUTAGENIC ACTIVATION OF CARCINOGENIC *N*-NITROSOPROPYLAMINES BY VARIOUS ANIMAL SPECIES AND MAN

Y. Mori<sup>1,3</sup>, H. Yamazaki<sup>1</sup> & Y. Konishi<sup>2</sup>

<sup>1</sup>Laboratory of Radiochemistry, Gifu Pharmaceutical University, 6-1, Mitahora-higashi 5-chome, Gifu 502, Japan; and

<sup>2</sup>Department of Oncological Pathology, Cancer Center, Nara Medical College, 840 Shijo-cho, Kashihara, Nara 634, Japan

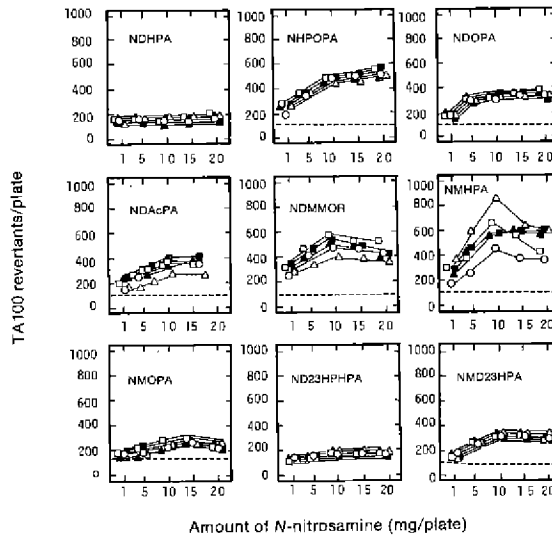
*N*-Nitrosomethyl(2-hydroxypropyl)amine (NMHPA), *N*-nitrosomethyl(2-oxopropyl)amine (NMOPA), *N*-nitrosomethyl(2,3-dihydroxypropyl)amine (NMD23HPA), *N*-nitrosobis(2-hydroxypropyl)amine (NDHPA), *N*-nitroso(2,3-dihydroxypropyl)(2-hydroxypropyl)amine (ND23HPHPA), *N*-nitrosobis(2-acetoxypopyl)amine (NDAcPA) and *N*-nitroso-2,6-dimethylmorpholine (NDMMOR) were mutagenic in *Salmonella typhimurium* strain TA100 in the presence of liver 9000 × *g* supernatant (S9) from mice, rats, hamsters, rabbits and monkeys. *N*-Nitroso(2-hydroxypropyl)(2-oxopropyl)amine (NHPOPA) and *N*-nitrosobis(2-oxopropyl)amine (NDOPA) were mutagenic in strain TA100 without metabolic activation, and both compounds were further activated by animal liver S9. NMOPA and NMHPA were also mutagenic in the presence of lung S9 from these animals and in the presence of human lung or liver S9. Pancreatic S9 from any of the animals did not activate any of the nine *N*-nitrosamines to mutagens.

NDHPA, NHPOPA, NDOPA, NMHPA, NMOPA, NDAcPA, ND23HPHPA, NMD23HPA and NDMMOR induce a high incidence of pancreatic tumours in hamsters, while the pancreas is not a target organ in rats, mice or rabbits, in which lung, liver or oesophageal carcinomas predominate (Lijinsky, 1984a; Konishi *et al.*, 1978b). NDHPA is an environmental contaminant of commercial samples of diisopropanolamine and triisopropanolamine (Issenberg *et al.*, 1984). In order to compare the capacity for tissue-specific metabolic activation in five animal species, including monkeys, and to allow estimation of the extent of cancer risk in humans, the mutagenic potential of nine *N*-nitrosopropylamines was examined in the presence of liver, lung or pancreatic S9 in the Ames preincubation assay.

Figure 1 shows the dose-response curves obtained in strain TA100 in the presence of animal liver S9. All the *N*-nitrosamines, except for NDHPA and ND23HPHPA, were mutagenic in the presence of liver S9 from the five animal species. NHPOPA and NDOPA were weakly mutagenic in strain TA100 without metabolic activation. NDHPA and ND23HPHPA were mutagenic in the presence of liver S9 from polychlorobiphenyl (PCB)-induced rodents (Mori *et al.*, 1986a). As shown in Figure 2, NMOPA was mutagenic in the presence of lung S9 from all the animals tested and from three humans (Fig. 2A,C), and NMHPA in the presence of lung S9 from PCB-induced rodents and of liver S9 from three humans (Fig. 2B,D). Lung S9 from animals or humans and human liver S9 had no effect on the activation of the other *N*-nitrosamines, and pancreatic S9 from PCB-induced animals did not activate any of the *N*-nitrosamines tested (Mori *et al.*, 1986b).

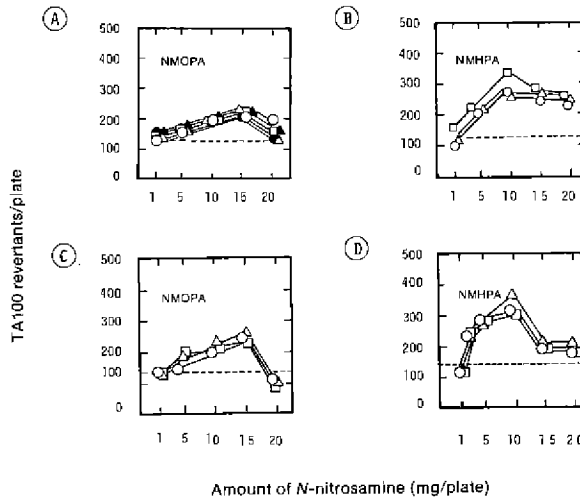
<sup>3</sup>To whom correspondence should be addressed

**Fig. 1. Mutagenicity of *N*-nitrosopropylamine derivatives in *S. typhimurium* TA100 in the presence of liver S9 from uninduced animals**



The curves represent the dose-response for mutagenic response in the presence of liver S9 from rats (○), hamsters (△), mice (□), rabbits (▲) and monkeys (■). Dotted lines indicate the number of spontaneous revertants.

**Fig. 2. Mutagenicity of NMOPA and NMHPA in *S. typhimurium* TA100 in the presence of animal and human lung or liver S9**



The curves represent the dose-response for mutagenic response: A, lung S9 from rats (○), hamsters (△), mice (□), rabbits (●) and monkeys (▲); B, PCB-induced lung S9 from rats (○), hamsters (△) and mice (□); C, human lung S9 from 40- (○), 45- (△) or 55- (□) year-old men; and D, human liver S9 from a 34-year-old man (○), and 34- (△) and 37- (□) year-old women. Dotted lines indicate the number of spontaneous revertants.

These results demonstrate a correlation between the known carcinogenicity of nine *N*-nitrosopropylamines in rats and hamsters and their mutagenicity when mediated by liver S9 from rats and hamsters, suggesting that *N*-nitrosopropylamines that are mutagenic in the presence of liver S9 from mice, rabbits and monkeys might also be carcinogenic in these animals. The results of the tissue-mediated mutagenicity assay suggest that metabolic activation of the nine *N*-nitrosamines by the liver may be necessary for pancreatic carcinogenesis in hamsters, and metabolism to NMOPA and NMHPA by the liver and further metabolism of both compounds by the lung may be important in lung carcinogenesis in other animal species. In-vivo administration of NDOPA, NHPOPA, NDHPA, NDMOR and other oxidative derivatives of *N*-nitrosodipropylamine to rats and hamsters led to methylation in the DNA or RNA of target organs, indicating formation of a potential methylating metabolite (Lawson *et al.*, 1981; Lijinsky, 1985). Accordingly, further studies of the metabolic activation of NMOPA and NMHPA by the lung are of interest in order to clarify the tissue-specific carcinogenic actions of *N*-nitrosopropylamine derivatives. The mutagenicity of NMOPA and NMHPA (Fig. 2) in the presence of human lung and liver S9, respectively, suggests that the compounds might act as alkylating agents in human lung and liver DNA. We have also found that microsomal cytochrome P450 in the liver and lung of humans and animals is involved in the mutagenic activation of nine *N*-nitrosopropylamines (Mori *et al.*, 1986a,b).

# METABOLISM OF THE OESOPHAGEAL CARCINOGEN *N*-NITROSOMETHYLAMYLAMINE: CHANGES WITH AGE, CLEARANCE FROM BLOOD AND DNA ALKYLATION

S.S. Mirvish<sup>1</sup>, C. Ji<sup>1</sup>, M. Makary<sup>1</sup>, H.A.J. Schut<sup>2</sup> & C. Krokos<sup>1</sup>

<sup>1</sup>*Eppley Institute for Research in Cancer,  
University of Nebraska Medical Center, Omaha, NE 68105, USA;*  
and <sup>2</sup>*Department of Pathology, Medical College of Ohio, OH, Toledo, 45208, USA*

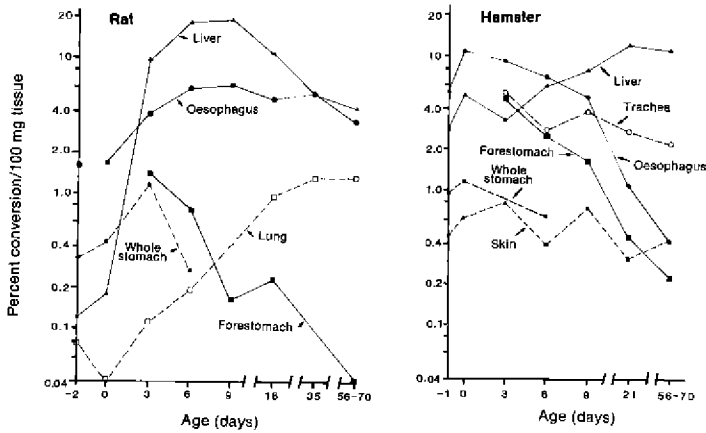
Freshly excised rat oesophagus and other tissues metabolize *N*-nitrosomethyl-*n*-amylamine (NMAA) to 2-, 3-, 4- and 5-hydroxy-NMAA (HO-NMAA), and 3- and 4-oxo-NMAA. We examined the development of this metabolism in MRC-Wistar rats and Syrian hamsters. In rats, oesophagus showed maximum metabolism at nine days of age, and forestomach showed considerable metabolism at three days, but none in adults. In newborn hamsters, oesophagus showed 10.4%; and in three-day hamsters, forestomach showed 4.9% metabolism, despite low or no metabolism by the adult tissues. Clearance of NMAA (25 mg/kg) from rat blood had a half-life of 21 min. The blood also contained considerable amounts of 4-oxo- and 4-HO-NMAA. Incubation of adult oesophagus and liver slices with <sup>3</sup>H-labelled NMAA produced DNA labelling, including labelled *N*7- and *O*<sup>6</sup>-methylguanine, with 14 times higher specific radioactivity in DNA from oesophagus than from liver. *O*<sup>6</sup>-Methylguanine was also measured by radioimmunoassay.

Our studies are designed to discover why NMAA induces tumours in the rat oesophagus but not in the histologically similar rat forestomach and only rarely in hamster oesophagus (Bulay & Mirvish, 1979, and unpublished results). Freshly removed adult rat oesophagus converted NMAA into 2-, 3- and 4-HO-NMAA in ratios of 0.32:1.00:0.85, together with smaller amounts of 3- and 4-oxo-NMAA and 5-HO-NMAA, whereas rat liver produced mainly 4-hydroxy-NMAA and rat lung, mainly 5-hydroxy-NMAA (Mirvish *et al.*, 1985, and unpublished results). Adult rat forestomach and adult hamster oesophagus showed much weaker or no NMAA metabolism. Hence, hydroxylation at positions 2 and 3 may predict NMAA carcinogenicity, which probably occurs *via* activation by  $\alpha$ -hydroxylation. This research was extended in three directions.

## Changes with age of NMAA metabolism in rats and hamsters

To help understand the species and tissue differences in NMAA metabolism, we examined it in tissues of rats and hamsters of various ages, from fetuses two days before term to young adulthood. Freshly removed tissues were incubated with NMAA, and the resultant metabolites were determined. The sum of the metabolites was expressed as percent yield from NMAA/100 mg tissue (Fig. 1).

In rats, metabolism by oesophagus reached a maximum of  $6.4 \pm 0.7\%$  (mean  $\pm$  SE) at nine days — about double the level of  $3.4 \pm 0.4\%$  in adult oesophagus. Metabolism in the forestomach reached  $1.4 \pm 0.3\%$  at three days, although this tissue showed no metabolism in adults. Metabolism by liver peaked at  $19.3 \pm 2.2\%$  at nine days — four times that in adult

**Fig. 1. Development of NMAA metabolism in rats and hamsters**

Freshly removed tissues from MRC-Wistar rats and Syrian hamsters were slit and rinsed with Eagle's medium (oesophagus, stomach and intestines) or cut into slices (other tissues), combined from several animals where needed, to give 50-100 mg tissue, and incubated for 3 h in 5 ml medium containing 3 mg NMAA. The medium was extracted with dichloromethane, and the extract was analysed by gas chromatography-thermal energy analysis, as described by Mirvish *et al.* (1985). Results for each metabolite (2-, 3-, 4- and 5-HO-NMAA and 3- and 4-oxo-NMAA) were expressed as percent yield/100 mg tissue. The figure (which has a logarithmic ordinate) shows the sum of all metabolites. Each point is the mean of 2-5 (mostly 3-5) experiments. For all points, the mean standard error was 13% of the mean values.

liver [similar to previous findings for *N*-nitrosodimethylamine metabolism (Davies *et al.*, 1976)]. Metabolism by lung increased steadily with age. In the newborn and/or three-day-old rat, no or < 0.01% metabolism was observed in the skin, small intestine, heart and spleen. Kidney showed 0.47% metabolism at three days.

In hamsters, metabolism in oesophagus reached  $10.6 \pm 1.4\%$  in newborn animals and then decreased to 0.4% in adults. Metabolism was also prominent in the forestomach, where it reached  $4.8 \pm 0.3\%$  at three days, and in skin. Metabolism in glandular stomach was 0.16% at three days and 0.01% at nine days. The trachea (the usual site of nitrosamine carcinogenesis in hamsters) had peak metabolism of  $4.7 \pm 0.4\%$  at three days and values of  $2.4 \pm 0.4\%$  at eight to ten weeks. Metabolism in the lungs was  $3.8 \pm 0.3\%$  at one day before birth, 1.8% at three days and 0.9% in adults.

Ratios between the metabolites were similar in the tissues of suckling and adult animals of both species, with some exceptions: rat and hamster forestomach and glandular stomach, hamster skin, hamster lung at -1 and +3 days, and adult hamster oesophagus all showed similar metabolite ratios to those in adult rat oesophagus (whereas adult rat lung produces mainly 5-HO-NMAA). The factors that regulate development of NMAA metabolism in each tissue of the two species remain to be determined. Prolonged contact of milk and/or food with the stomach wall might repress NMAA-metabolizing cytochrome P450 isozymes in this tissue, but not in oesophagus. (Rats are weaned at 12-21 days.) The low activity in adult hamster oesophagus compared to adult rat oesophagus could be associated with the early peaking of this metabolism in hamsters.

### Clearance of NMAA from the blood of rats

To help evaluate our studies of carcinogenesis and metabolism, we examined the clearance of NMAA from the blood of four 280-g male MRC-Wistar rats kept on standard laboratory chow. NMAA (25 mg/ml water/kg) was injected intraperitoneally, and blood samples (75  $\mu$ l) were taken in heparinized tubes from the orbital sinus. Each sample was mixed with 0.5 ml water and then 0.5 ml of 12N hydrochloric acid, and extracted for 30 min with 6 ml dichloromethane. The dichloromethane phase was dried, concentrated to 0.1 ml and analysed by gas chromatography-thermal energy analysis (GC-TEA). Recoveries of 3- $\mu$ g samples added to blood were 50-100% for NMAA and its metabolites, and results were corrected for these recoveries. The mean (standard error/mean value) was 20% for NMAA and 8% for 4-oxo-NMAA.

The NMAA level reached 11.2  $\mu$ g/ml blood at 15 min. Computer analysis showed that NMAA elimination followed one-compartment first-order kinetics, with a half-life of 21 min. The major blood metabolites were 4-oxo-NMAA (maximum, 2.2  $\mu$ g/ml at 1-2 h) and 4-HO-NMAA (maximum, 1.9  $\mu$ g/ml at 30 min). The area under the 4-oxo-NMAA curve, i.e., exposure to 4-oxo-NMAA, was about one-half of that for NMAA. Of the urinary metabolites, 80% were excreted in the first 6 h.

### DNA alkylation by NMAA

We obtained tritium-labelled NMAA. The labelling positions are unknown, although we expect it to have occurred predominantly at the methyl and amyl-1-C positions, which readily undergo hydrogen exchange. (This was shown in deuterium exchange studies.) The  $^3$ H-NMAA was incubated with rat tissues *in vitro*; DNA was isolated and its hydrolysate was analysed by high-performance liquid chromatography for apurinic acid, and *N7*-methylguanine and *O6*-methylguanine. Table 1 shows that (i) specific radioactivity of the DNA was 14 times higher in oesophagus than in liver, and (ii) the mean content of *O6*-methylguanine (as dpm/ $\mu$ g DNA) was 19 times higher for oesophagus than for liver. This helps explain why NMAA induces oesophageal and not liver cancer in rats. The mean *O6*-methylguanine yield in the oesophagus of 5.3 dpm/ $\mu$ g DNA corresponds to 56 pmol *O6*-methylguanine/mg DNA, if we assume that all  $^3$ H in NMAA was in the methyl group and all  $^3$ H in the methyl group was transferred to *O6*-methylguanine. These assumptions give the maximum possible value.

**Table 1. Radioactivity in DNA and its hydrolysis products when  $^3$ H-NMAA was incubated with fresh tissues of adult male MRC-Wistar rats**

Tissue	No. of experiments	Total counts (dpm/ $\mu$ g DNA)	Mean percent dpm in			<i>O6</i> -meGua (dpm/ $\mu$ g DNA)
			AP	<i>N7</i> -meGua	<i>O6</i> -MGmeGua	
Oesophagus	4	89 $\pm$ 58	43	51	6.0	5.3
Liver	4	6.4 $\pm$ 4.6	70	26	4.4	0.28

$^3$ H-NMAA was labelled by tritium exchange at Amersham Corporation to give 15 mCi  $^3$ H-NMAA, with 0.67  $\mu$ Ci/ $\mu$ g. Adult rat tissues (six slit oesophagi or 500 mg liver slices) were incubated with 100  $\mu$ Ci (300  $\mu$ g) NMAA/10 ml Eagle's medium for 5 h at 37°C. DNA was isolated by a phenol procedure (Bogden *et al.*, 1981), assayed for DNA content and radioactivity, hydrolysed (0.1N hydrochloric acid, 0.5 h, 70°C) and subjected to reversed-phase high-performance liquid chromatography on a C-18 column, which was eluted with 0.01N phosphate buffer:methanol 4:1, pH 5.6 (Bogden *et al.*, 1981). Eluate fractions were assayed for radioactivity.

In preliminary studies, fresh rat oesophagi were incubated with 3 mg/l unlabelled NMAA for 3 h. DNA was isolated by hydroxyapatite chromatography (Stoner *et al.*, 1982), hydrolysed enzymically to nucleosides, and assayed for *O*<sup>6</sup>-methylguanosine by double-antibody radioimmunoassay (Wild *et al.*, 1983). Two oesophagi showed 16 and 24 pmol *O*<sup>6</sup>-methylguanosine/mg DNA, similar to the results obtained under different conditions using <sup>3</sup>H-NMAA.

#### Acknowledgements

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# EXHALATION OF *N*-NITROSOETHYLVINYLAMINE AFTER APPLICATION OF *N*-NITROSODIETHYLAMINE TO SPRAGUE-DAWLEY RATS

R.G. Klein, B. Schmezer, P. Schmezer & B. Spiegelhalder

*Institute for Toxicology and Chemotherapy,  
German Cancer Research Center, Im Neuenheimer Feld 280, 6900 Heidelberg,  
Federal Republic of Germany*

Our method of endotracheal intubation makes it possible to collect exhaled air directly from the respiratory tract, thus eliminating the possibility of artefact formation and decomposition of metabolites. *N*-Nitrosodiethylamine (NDEA) has been postulated as a precursor of *N*-nitrosoethylvinylamine (NEVA), however, NEVA has not been detected as a metabolite of NDEA. Following endotracheal intubation and intravenous application of 550  $\mu\text{g}$  NDEA to Sprague-Dawley rats, appreciable amounts of NEVA and unaltered NDEA were found in exhaled air. Further confirmation that NEVA is a metabolite of NDEA was obtained when, after eliminating oxidative decomposition of the nitrosamine with disulfiram in an enzyme inhibition assay, pretreated rats exhaled only traces of NEVA. Such findings could be informative with respect to the organotropism of nitrosamine carcinogenesis.

Exposure to *N*-nitrosamines has been shown to occur in certain industrial work places (Spiegelhalder & Preussmann, 1982). The major route of absorption of these substances into the organism is probably by inhalation. As a contribution to human risk estimates and in view of the multipotent organotropism of *N*-nitrosamine carcinogenesis in animals, it is necessary to study inhalation and exhalation of these compounds quantitatively.

With our method of endotracheal intubation (Klein & Schmezer, 1984) under Thalamonal narcosis (Droperidol/Fentanyl, H. Janssen, Düsseldorf, FRG), inhalation of volatile substances proceeds directly into the trachea and the lungs without being disturbed by absorption into the nose or upper respiratory tract of the animals. Additionally, we are able to collect the exhaled air directly from the respiratory tract. Thus, the possibility of artefact formation and/or subsequent decomposition of metabolites may be eliminated.

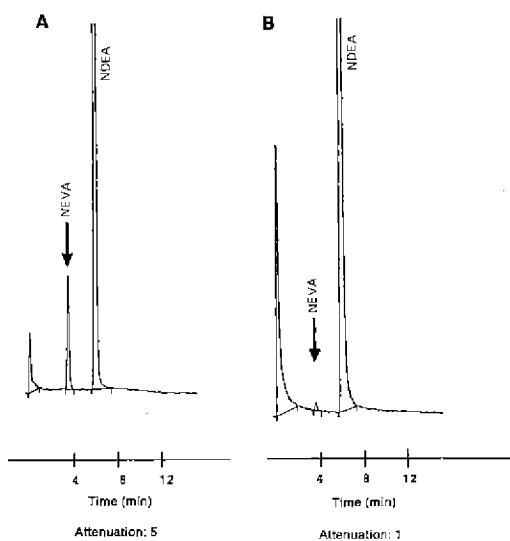
Female Sprague-Dawley rats (Medizinische Hochschule, Hanover, FRG), 250-300 g body weight, were anaesthetized with Thalamonal in order to allow positioning of an endotracheal tube. Then, 1 ml NDEA (550  $\mu\text{g}$  or 5.5 mg in saline) was injected into the tail-vein within 10 min; for a further 60 min, the animal was joined to an exhalation apparatus in which the *N*-nitrosamines were collected in a Thermosorb tube. Besides NDEA, NEVA was found in a 4.3% yield ( $n=5$ ; mean,  $4.3 \pm 0.5\%$ ). The presence of NEVA, as determined by gas chromatography-thermal energy analysis with internal standard was confirmed by mass spectrometry.

In another experiment, rats were fasted for two days in order to induce *N*-nitrosamine metabolism (Tu & Yang, 1983). No increase in NEVA exhalation, however, was observed in comparison to the control rats without fasting, as described above.



Confirmation of NEVA as a metabolite of NDEA was obtained when the oxidative decomposition of the nitrosamine was inhibited by concomitant administration of disulfiram in an enzyme inhibition assay (Schmähl *et al.*, 1976b; Frank *et al.*, 1980; Frank & Wiessler, 1986a). Disulfiram (500 mg/kg) was administered by gavage as a suspension in 4% starch solution; rats received 0.5 ml of the suspension per 100 g body weight. Only traces of NEVA could be detected in the exhaled air of these animals (Fig. 1; the first peak is the solvent peak, which is enhanced in the second chromatogram because of higher amplification of the detector. Even under these circumstances, the NEVA peak is very small in comparison).

**Fig. 1.** Gas chromatography-thermal energy analysis chromatogram of NEVA and NDEA exhaled after injection of 550  $\mu$ g NDEA alone (A) and after pretreatment with disulfiram (B)



Experimental data and biochemical considerations led Krüger (1971) and Krüger and Bertram (1973) to conclude that  $\beta$ -oxidation is a possible metabolic conversion of aliphatic *N*-nitrosodialkylamines. Althoff *et al.* (1977) and Green and Althoff (1982) postulated that some of the intermediate metabolites formed during *N*-nitrosamine activation may be  $\alpha\beta$ -unsaturated derivatives, before further oxidation occurs. They presumed that NEVA could arise as an intermediate of NDEA. Our experiments have shown for the first time that NEVA is indeed formed during NDEA metabolism.

These findings could be helpful in explaining the mechanisms of the organotropism of *N*-nitrosamine carcinogenesis. Druckrey *et al.* (1967) showed that 90% of the malignant neoplasms in rats caused by NEVA are oesophageal tumours. In contrast, NDEA induces mainly liver tumours and a few oesophageal tumours. Thus, the observed oesophageal tumours in rats induced by NDEA may be a result of NEVA formation.

#### Acknowledgements

We thank Mrs H. Oberst for excellent technical help and Mr G. Würtele for carrying out the analysis.

# INVESTIGATION INTO THE PHARMACODYNAMICS OF THE CARCINOGEN *N*-NITRODIMETHYLAMINE

M. Hassel, E. Frei, H.R. Scherf & M. Wiessler

*German Cancer Research Center,  
Im Neuenheimer Feld 280, Heidelberg, Federal Republic of Germany*

*N*-Nitrodimethylamine (NTDMA) was found to be a carcinogen of the nasal mucosa leading to aesthesioneuroepitheliomas in BDVI rats. *N*-Nitromethylamine (NTMA), a product of the oxidative metabolism of NTDMA, was also carcinogenic, leading to neurogenic tumours of the lumbar region of the spine. The 100 000 × *g* supernatant of both liver and nasal mucosa contains an enzyme capable of reducing NTDMA to *N*-nitrosodimethylamine (NDMA). In the microsomal fraction of both organs, NTDMA is oxidized to formaldehyde. The fractions from nasal mucosa have a higher capacity than the corresponding liver fractions to both oxidize and reduce NTDMA. NDMA was detected in blood and urine from rats treated with NTDMA. The elimination of NTDMA from blood occurs biphasically, with an initial half-life of 3.5 min.

The biological properties of NTDMA, an oxidation product of NDMA, include its mutagenicity to *Salmonella typhimurium* TA100 at high concentrations after metabolism by a rat liver fraction. The stable metabolite after oxidative dealkylation is NTMA, which is not mutagenic. The biologically active metabolite in this bacterial system was found to be formaldehyde (Frei *et al.*, 1984). The reported carcinogenicity of NTDMA (for a list of references, see Pool *et al.*, 1984a) is compared with that of NTMA, and possible activation pathways for both compounds are investigated.

## Carcinogenicity of NTDMA and NTMA

Both compounds exert high organ specificity (Table 1) and induce neurogenic tumours, but in different organs. Lower doses of NTDMA led not only to aesthesioneuroepitheliomas but also to tumours of the lumbar region of the spine; thus, an oxidative metabolism of NTDMA leading to NTMA may be postulated.

## Oxidative metabolism of NTDMA in target and nontarget organs

The *C*-hydroxylation of NTDMA to yield formaldehyde and NTMA was compared in liver microsomes and microsomes from nasal mucosa of untreated male and female Sprague-Dawley rats. Both males and females had a higher capacity to metabolize NTDMA in the nose than in the liver. The incubation mixture contained 3 μmol NTDMA per 2 mg liver microsomal protein (as described by Bertram *et al.*, 1982, but without semicarbazide hydrochloride) and 0.6 μmol NTDMA per 0.4 mg microsomal protein from nasal mucosa. In male rats, 14.5 nmol formaldehyde per mg protein were released from liver microsomes and 41.8 nmol from nasal mucosa; in female rats, 12.9 nmol and 31.9 nmol were released, respectively. The enzyme was inhibited competitively by NDMA in the liver. The target organ (nose) thus has a greater capacity to metabolize NTDMA oxidatively than the

**Table 1. Numbers of animals with tumours and their locations**

Treatment <sup>a</sup>	Sex	Dosage ( $\mu\text{mol/kg}$ once/ week)	Median survival time (days)	Numbers of animals with tumours				
				Nasal cavity	Spinal cord	Spinal nerves	Peripheral nerves	Other sites <sup>b</sup>
NTDMA	M	0	790	-	-	-	-	5(803)
NTDMA	M	0.5	697	6(712)	2(742)	2(749)	-	3(658)
NTDMA	M	1.0	446	10(446)	-	-	-	-
NTDMA	F	0	896	-	-	-	-	9(907)
NTDMA	F	0.5	729	3(804)	-	1(633)	-	8(729)
NTDMA	F	1.0	451	8(465)	-	1(441)	-	3(519)
NTMA	M	0	730	-	-	-	-	7(789)
NTMA	M	0.5	725	-	2(701)	5(792)	2(654)	3(780)
NTMA	M	1.0	612	-	8(607)	4(652)	2(624)	2(652)
NTMA	F	0	775	-	-	-	-	8(775)
TMA	F	0.5	814	-	-	3(869)	2(838)	8(838)
NTMA	F	1.0	653	-	5(687)	1(646)	-	8(622)

<sup>a</sup>Ten BDVI rats per group were treated once weekly with 1  $\mu\text{mol/kg}$  or 0.5  $\mu\text{mol/kg}$  per gavage of NTDMA or NTMA. Numbers of animals dying with tumours are given with the median survival time in days in parentheses.

<sup>b</sup>Occurrence of tumours at 'other sites' was not related to the dose of carcinogen applied.

nontarget organ (liver), resulting in formaldehyde and NTMA as metabolites. Formaldehyde is a known nasal carcinogen (Albert *et al.*, 1982), but it induces squamous-cell carcinoma and not aesthesioneuroepitheliomas as does NTDMA. NTMA is also a carcinogen but probably requires further metabolism as it is not a very reactive compound. *In vitro* we could detect no oxidative metabolism of NTMA.

Another possible mechanism for the activation of NTDMA could be its reduction to NDMA which, after further oxidative metabolism, would be a potent carcinogen.

#### Anaerobic metabolism of NTDMA

The soluble fraction (100 000  $\times g$  supernatant) of both nasal mucosa and liver homogenate contained a reductase capable of reducing NTDMA to NDMA. The reduction occurred without addition of reduced purine phosphates and was enhanced by both NADH and an NADPH regenerating system. The incubation assays were performed like the aerobic assays, but containers were bubbled with argon for 5 min prior to adding the substrate.

The rate of reduction of NTDMA to NDMA in the nasal cytosol was more efficient than oxidation in microsomes: 1.9  $\mu\text{mol}$  NTDMA/mg protein resulted in 28 nmol NDMA. The rate of reduction of NTDMA/mg protein in liver cytosol was of the same order as the oxidation in the microsomal fraction. The liver enzyme appeared to be inhibited at substrate concentrations greater than 326 nmol/mg protein (12.0 nmol NDMA recovered at this concentration). A higher rate of reduction can be assumed to occur in the whole organ, which contains five times more cytosolic protein than microsomal protein. For the nasal mucosa, this would imply a three-fold higher rate of reduction than oxidation in the intact organ.

The resulting NDMA could be activated oxidatively and might ultimately lead to the aesthesioneuroepitheliomas that are observed after inhalation of NDMA (Druckrey *et al.*, 1967). Nasal microsomes from some species show an even higher rate of oxidative metabolism of nitrosamines than liver microsomes (Dahl, 1985).

The liver enzyme was partly purified by fractionated precipitation with ammonium sulfate (55-65%) and DEAE cellulose chromatography with a 0.01-0.3 M phosphate buffer gradient at pH 6.4 (modified from Ernster *et al.*, 1962). 'NTDMA reductase' eluted in the first peak with a five-fold higher specific activity than the starting material. Only NADH could act as a coenzyme for this enriched fraction.

Commercially available reductases such as xanthine oxidase and DT diaphorase with lipamide dehydrogenase (EC1.6.4.3), could not reduce NTDMA to NDMA at pH 7.4.

### Pharmacokinetics of NTDMA

Male Sprague-Dawley rats were treated intravenously with 2 mg NTDMA per kg, and blood was drawn from the catheterized jugular vein 1-60 min after NTDMA administration. In another experiment, unanaesthetized rats were killed 5, 10, 30 and 40 min after NTDMA administration, and blood (100-200  $\mu$ l) was extracted and analysed for NTDMA and NDMA by gas chromatography-thermal energy analysis (Eisenbrand *et al.*, 1983). No difference was detected between the two methods of obtaining blood. The curves showed a biphasic character, with a reproducible increase in NTDMA 5-10 min after administration and another increase after 15-30 min. The half-life of the first elimination phase was 3.5 min. In two animals, NDMA was detectable in blood 2 min after NTDMA administration and again after 8 min, concomitant with the increase in NTDMA blood levels. The concentration of NDMA was 0.17-0.3% of the NTDMA content in the blood at the respective time points.

In anaesthetized animals (Rompun and Ketanest or diethylether) treated with more than 10 mg/kg NTDMA, both NDMA and unchanged NTDMA were found in the urine. The absolute amount of both compounds varied considerably between individual animals, but NDMA was always detected. Ether is a known inhibitor of NDMA oxidation *in vivo* (Spiegelhalder *et al.*, 1982); without anaesthesia, NDMA was detected after administration of 90 mg/kg NTDMA but not after administration of 10 or 2 mg/kg NTDMA.

The pharmacokinetics of NTDMA are therefore very complex, the intact molecule being detectable as long as 2 h after intravenous administration. Reduction of NTDMA to the potent carcinogen NDMA occurs *in vivo* as well as *in vitro*. NTMA was not detectable *in vivo* after administration of NTDMA, as this compound is very difficult to analyse by gas chromatography-thermal energy analysis or high-performance liquid chromatography. The metabolism of the compound *in vitro* is under investigation. A reductive pathway would lead directly to the highly reactive methyldiazonium ion, which could react with nucleophiles at the site of its genesis.

The striking ability of these two nitramines to transform neurogenic tissues specifically in different organs cannot be explained by the results of the experiments described above. It is furthermore remarkable that, despite the presence of NDMA resulting from NTDMA, no tumour 'typical' for NDMA (liver, kidney) occurred. The competitive inhibitor NTDMA may suppress the activation of NDMA to a carcinogen in the liver. The finding that mammals are capable of reducing aliphatic nitro compounds (in the above experiments, to yield carcinogenic *N*-nitroso compounds) is new and may help to understand the fate of such compounds in the human body.

### Acknowledgements

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# URINARY METABOLITES OF *N*-NITROSODIBUTYLAMINE IN THE RAT: IDENTIFICATION OF *N*-ACETYL-*S*-BUTYL-L-CYSTEINE DERIVATIVES

E. Suzuki<sup>1</sup>, M. Osabe<sup>1</sup>, M. Okada<sup>1</sup>, T. Ishizaka<sup>2</sup> & T. Suzuki<sup>2</sup>

<sup>1</sup>Tokyo Biochemical Research Institute, Takada 3-41-8,  
Toshima-ku, Tokyo 171; and <sup>2</sup>Division of Food, National Institute of  
Hygienic Sciences, Kamiyoga 1-18-1, Setagaya-ku, Tokyo 158, Japan

*N*-Acetyl-*S*-(butyl, 3-oxobutyl and 3-hydroxybutyl)-L-cysteines have been isolated and identified (as their methyl esters) from the urine of rats given *N*-nitrosodibutylamine (NDBA), *N*-nitrodibutylamine (NTDBA) and their corresponding  $\alpha$ -acetoxy derivatives, *N*-nitroso-*N*-butyl(1-acetoxybutyl)amine and *N*-nitro-*N*-butyl(1-acetoxybutyl)amine, respectively. Greater amounts of these L-cysteine derivatives were detected in urine after administration of NDBA than of NTDBA. This suggests that the markedly different biological activities of NDBA and NTDBA might be due, in part, to a difference in their alkylating abilities *in vivo*.

NDBA is a potent carcinogen in rats and other animal species, while NTDBA, the corresponding *N*-nitramine, is a weak carcinogen (Pliss *et al.*, 1982). In order to elucidate the markedly different biological activities of these two compounds, a comparative study on *in-vivo* alkylation of glutathione by these compounds was performed. Urinary *N*-acetyl-*S*-alkyl-L-cysteines were used as a marker for *in-vivo* alkylation by these compounds, since these cysteines are considered to be formed by degradation and subsequent acetylation of *S*-alkylglutathiones produced by *S*-alkylation of glutathione.

## Isolation and identification of *N*-acetyl-*S*-alkyl-L-cysteines in rat urine

In order to identify *N*-acetyl-*S*-alkyl-L-cysteines by mass spectrometry, the L-cysteine derivatives were isolated from rat urine and partially purified as follows: ethyl acetate extraction of urine at pH 1; derivatization of the extract with excess diazomethane; washing of the derivatized extract with water; and chromatography on silica gel by successive elution with chloroform (fraction A) and mixtures of methanol:chloroform in a ratio of 1:9 (fraction B) and 1:9 (fraction C).

The L-cysteine derivatives were determined by gas chromatography (GC) in fraction A, fraction B and fraction C for the methyl esters of *N*-acetyl-*S*-(butyl and *sec*-butyl)-L-cysteines, *N*-acetyl-*S*-(2- and 3-oxobutyl)-L-cysteines and *N*-acetyl-*S*-(2-, 3- and 4-hydroxybutyl)-L-cysteines, respectively. Experimental details of the synthesis of these L-cysteine derivatives (except *N*-acetyl-*S*-(*sec*-butyl)-L-cysteine) have been reported previously (Suzuki *et al.*, 1984). Their chromatographic profiles were compared with those of the compounds in the three fractions. GC with a 1% OV 225 column was performed in the S mode with a Shimadzu GC-9AM equipped with a flame photometric detector. The mass spectra of authentic methyl esters of the L-cysteine derivatives and those of the GC peaks

eluting at the retention times of the corresponding authentic samples in the chromatogram of the purified urine extracts (fractions A, B and C) were compared. Mass spectra were obtained by coupled GC-mass spectrometry on a Shimadzu LKB-9000 mass spectrometer in the electron impact mode.

### In-vivo alkylation by NDBA and *N*-nitroso-*N*-butyl(1-acetoxybutyl)amine and by their corresponding *N*-nitramines

*N*-Acetyl-*S*-butyl-L-cysteine, *N*-acetyl-*S*-(3-oxobutyl)-L-cysteine and *N*-acetyl-*S*-(3-hydroxybutyl)-L-cysteine were isolated and identified (as their methyl esters) in the urine of rats given NDBA (Table 1). *N*-Acetyl-*S*-(*sec*-butyl)-L-cysteine, *N*-acetyl-*S*-(2-oxobutyl)-L-cysteine and *N*-acetyl-*S*-(4-hydroxybutyl)-L-cysteine were not detected in the urine, and *N*-acetyl-*S*-(2-hydroxybutyl)-L-cysteine was detected in only a trace amount. After intraperitoneal administration, greater urinary excretion of the three L-cysteine derivatives was observed with 1-acetoxy NDBA (NBACBA) than with NDBA. After oral administration, much less *N*-acetyl-*S*-butyl-L-cysteine was excreted with NBACBA. No *N*-acetyl-*S*-butyl-L-cysteine was detected after intraperitoneal administration of either NTDBA or 1-acetoxy NTDBA (NTBACBA), and a much lower excretion of the three L-cysteine derivatives was observed after oral administration of NTDBA.

**Table 1. Urinary excretion<sup>a</sup> of *N*-acetyl-*S*-alkyl-L-cysteines in rats after administration of NDBA, NTDBA and related compounds**

Compound	Effective no. of animals	Method of administration	Dose (mg/rat)	Urinary <i>N</i> -acetyl- <i>S</i> -alkyl-L-cysteine (% of dose)				
				Butyl (Bu)	3-oxo-Bu	3-HO-Bu	3-oxo-Bu: 3-HO-Bu	Total <sup>b</sup>
NDBA	2	Intra-peritoneal	50	0.37	0.45	0.09	83 : 17	0.91
NBACBA	4	Intra-peritoneal	25	0.47±0.07 <sup>c</sup>	1.10±0.18	0.23±0.03	82 : 18	1.81
NTDBA	2	Intra-peritoneal	50	ND <sup>d</sup>	0.20	0.05	80 : 20	0.25
NTBACBA	3	Intra-peritoneal	25	ND	0.49±0.03	0.09±0	84 : 16	0.58
NDBA	5	Oral	55-74	0.64±0.09	1.03±0.13	0.09±0.02	92 : 8	1.76
NBACBA	6	Oral	10-85	0.002±0.003	1.06±0.20	0.11±0.03	91 : 9	1.17
NTDBA	3	Oral	78-86	0.003	0.09±0.02	0.01±0	90 : 10	0.10

<sup>a</sup>Four-(intra-peritoneal) or three-day (oral) urine samples were collected with protection from light at 24-h intervals. Male Sprague-Dawley rats weighing 235-285 g were used. After an overnight fast, the test compound was administered to animals by gastric intubation without vehicle or intra-peritoneally in corn oil.

<sup>b</sup>Sum of three *N*-acetyl-*S*-alkyl-L-cysteines

<sup>c</sup>Mean ± SD

<sup>d</sup>Not detectable (< 0.001%)

The metabolic pattern and the metabolic oxidation rate of NDBA and NTDBA *in vitro* in rat liver preparations were similar, and both compounds were activated to mutagenic compound(s) through  $\alpha$ -hydroxylation (Suzuki *et al.*, 1983b, 1985). No essential difference between NDBA and NTDBA was seen in the metabolic pattern *in vivo* in rats (Suzuki & Okada, 1980; Suzuki *et al.*, 1986). However, NTDBA had much less mutagenic activity than NDBA (Suzuki *et al.*, 1985), in accordance with the difference in their carcinogenic potency. Since the mutagenic activity of these compounds is considered to be mediated through alkylation of cellular macromolecules, the alkylation of glutathione *in vivo* was studied, using urinary *N*-acetyl-*S*-butyl-L-cysteine derivatives as a marker.

The ability of NDBA to alkylate glutathione *in vivo* was much greater than that of NTDBA, especially when they were administered by the oral route. This suggests that the markedly different biological activities of NDBA and NTDBA might be due, in part, to a difference in their alkylating abilities *in vivo*. The ratios of *N*-acetyl-*S*-(3-oxo- and 3-hydroxy-butyl)-L-cysteines are constant, depending on the route of application of the compounds, indicating a possible equilibrium between these two metabolites, as reported by Suzuki *et al.* (1984).

The alkylating species produced from NDBA *in vitro* is considered to be a butyl cation generated by  $\alpha$ -hydroxylation, on the basis of the formation of both *sec*- and *n*-butyl alcohol (Suzuki *et al.*, 1983b). However, production of *N*-acetyl-*S*-(*sec*-butyl)-L-cysteine from NDBA could not be demonstrated *in vivo*. Only a trace amount of *N*-acetyl-*S*-butyl-L-cysteine was detected after oral administration of NBaCBA (a model compound of  $\alpha$ -hydroxy NDBA). This may be due to differential stability of the compound depending on the route of administration.

*N*-Acetyl-*S*-methylcysteine was detected in the urine of rats given *N*-nitrosodimethylamine (Hemminki, 1982). If determination of carcinogen-adducts in urine is an important method for monitoring chemical exposure, the analysis of urinary *N*-acetyl-*S*-alkyl-L-cysteines may be useful for monitoring human exposure to various alkylating agents.

## EXTRAHEPATIC MICROSOMAL METABOLISM OF *N*-NITROSODI-*n*-BUTYLAMINE IN RATS

E. Richter, J. Schulze & W. Zwickenflog

*Walther Straub-Institut für Pharmakologie und Toxikologie,  
Ludwig-Maximilians-Universität München, Nussbaumstrasse 26,  
D-8000 München 2, Federal Republic of Germany*

The  $\omega$ - and  $\omega$ -1-hydroxylation of *N*-nitrosodi-*n*-butylamine (NDBA) has been studied in microsomes from rat liver, lung, intestine and kidney. Both reactions followed at least two enzyme kinetics with low (2-10  $\mu$ M) and high (1 mM)  $K_m$  values. Whereas  $\omega$ -1-hydroxylation was the predominant pathway in liver,  $\omega$ -hydroxylation was more important in extrahepatic tissues. First-pass metabolism of NDBA in lungs and intestinal mucosa may be of importance in the development of urinary bladder tumours in rats.

It has been shown that  $\omega$ -oxidation of NDBA to *N*-nitrosobutyl(4-hydroxybutyl)amine (NB4HBA) and subsequently to *N*-nitrosobutyl(3-carboxypropyl)amine is responsible for its tumorigenic action on the urinary bladder (Okada & Ishidate, 1977). However, in rat-liver preparations,  $\alpha$ - and  $\omega$ -1-oxidation are the predominant pathways (Janzowski *et al.*, 1982a; Suzuki *et al.*, 1983). In contrast, in microsomes of small-intestinal mucosa, NB4HBA is the major metabolite formed (Pacifci *et al.*, 1986).

### Isolation of microsomes and incubation with NDBA

Female Sprague-Dawley rats (180-240 g) were killed by cervical dislocation. The proximal 30 cm of jejunum were excised and the mucosal cells isolated by the vibration/EDTA-chelating procedure (Borm *et al.*, 1983). The mucosal cells, as well as livers, kidneys and lungs, of five animals each were homogenized in five volumes of 0.25 M sucrose (pH 7.4 with Tris). After sequential centrifugation at  $9000 \times g$  and  $105\,000 \times g$ , the ensuing pellet was resuspended in 0.15 M phosphate buffer (pH 7.4). Then, 0.1-1.5 mg protein were incubated at 37°C with 0.5-1000  $\mu$ M/1 [1- $^{14}$ C]-NDBA in the presence of 10 mM magnesium chloride and an NADPH regenerating system. The reaction was stopped after 10-30 min by addition of 0.2 ml 10% trichloroacetic acid, centrifuged and the clear supernatant analysed using an on-line high-performance liquid chromatography precolumn technique (Pacifci *et al.*, 1986). NDBA and its major metabolites, NB4HBA and *N*-nitrosobutyl(3-hydroxybutyl)amine (NB3HBA), were separated on an analytical column (20 + 125 mm length, 4.6 mm i.d., filled with Nucleosil 100, C18, 5 $\mu$ , Gynkotek, München, FRG) using a gradient with 10-80% acetonitrile in buffer pH 7. Detection was by radioactivity monitoring (Ramona, Isomess, Straubenhardt, FRG).

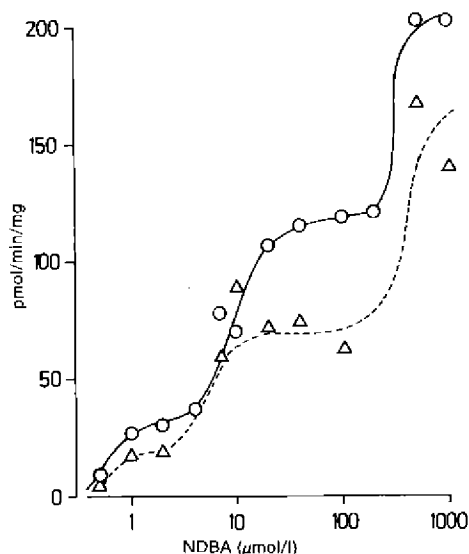
Apparent  $K_m$  and  $V_{max}$  values were calculated on a microcomputer using a nonlinear least-squares program (MULTI; Yamaoka *et al.*, 1981).



### Hydroxylation of NDBA in microsomes of rat liver, lung, kidney, and small intestinal mucosa

No metabolism of NDBA was observed in the absence of either NADPH or microsomal protein. Gassing the incubation mixture with 80% carbon monoxide in oxygen or addition of *para*-chloromercuribenzoate (1 mM) resulted in a 98.5% inhibition, whereas potassium cyanide (10 mM) did not affect NDBA hydroxylation. As illustrated in Figure 1, NDBA hydroxylation to NB4HBA and NB3HBA followed two enzyme kinetics, one with a very low and another with a high apparent  $K_m$  value (Table 1). The high  $K_m$  value could not be determined exactly. Because of the limited solubility of NDBA in the aqueous incubation medium, concentrations higher than 1 mM could not be used. Whereas  $\omega$ -1-hydroxylation was the predominant pathway in liver,  $\alpha$ -hydroxylation was more important in all other organs, especially at low NDBA concentrations.

**Fig. 1. Hydroxylation of NDBA to NB4HBA (○) and NB3HBA (Δ) in microsomes of isolated small-intestinal mucosal cells**



Multiple  $K_m$  values have been reported for other nitrosamines (Archer & Labuc, 1985). Further studies will be directed at the identification of different isozymes of cytochrome P450 involved in NDBA metabolism.

In humans exposed to low concentrations of NDBA in air (Spiegelhalder & Preussmann, 1983a) and/or food (Havery & Fazio, 1985), extrahepatic first-pass metabolism in lungs and small intestine (Richter *et al.*, 1986) may play a prominent role, yielding high amounts of NB4HBA, a precursor of the proximate bladder carcinogen, *N*-nitrosobutyl(3-carboxypropyl)-amine (Okada & Ishidate, 1977).

#### Acknowledgements

We thank Dr M. Wiessler for providing us with  $^{14}\text{C}$ -NDBA. We also wish to thank Miss B. Wankel for technical help. This work was supported by the Deutsche Forschungsgemeinschaft Grant No. Ri 395/2-2.

**Table 1. Apparent kinetic parameters of microsomal hydroxylation of NDBA**

Organ		NB4HBA		NB3HBA	
		$K_m$ ( $\mu\text{mol}$ )	$V_{\text{max}}$ ( $\text{pmol}/\text{min}/\text{mg}$ )	$K_m$ ( $\mu\text{mol}$ )	$V_{\text{max}}$ ( $\text{pmol}/\text{min}/\text{mg}$ )
Intestine	low	$5.6 \pm 1.8^a$	$113 \pm 23$	$6.3 \pm 2.2$	$83 \pm 17$
	high	$> 1000^b$	—	$> 1000$	—
Lung	low	$2.7 \pm 1.8$	$730 \pm 290$	$1.8 \pm 1.9$	$365 \pm 215$
	high	$350 \pm 550$	$3500 \pm 3000$	$147 \pm 188$	$1650 \pm 790$
Kidney	low	$2.3 \pm 0.8$	$153 \pm 25$	$0.6 \pm 0.7$	$87 \pm 16$
	high	$> 1000$	—	$> 1000$	—
Liver	low	$12 \pm 11$	$76 \pm 63$	$2.8 \pm 0.7$	$500 \pm 87$
	high	$> 1000$	—	$> 1000$	—

<sup>a</sup>Mean  $\pm$  SD calculated by MULTI (Yamaoka *et al.*, 1982) from the results of the incubation of a single batch of microsomes with 10-13 different NDBA concentrations (0.5-1000  $\mu\text{M}$ ) using the following equation:

$$V = \frac{V_{\text{max}_1}^{\text{app}} \times C}{K_{m_1}^{\text{app}} + C} + \frac{V_{\text{max}_2}^{\text{app}} \times C}{K_{m_2}^{\text{app}} + C}$$

where  $V$  = rate of formation of NB4HBA or NB3HBA (nmol/min/mg microsomal protein) and  $C$  = NDBA concentration ( $\mu\text{mol}$ )

<sup>b</sup> $K_m$  value higher than the highest NDBA concentration tested

## EXPERIMENTAL MODEL FOR INVESTIGATING BLADDER CARCINOGEN METABOLISM USING THE ISOLATED RAT URINARY BLADDER

L. Airoidi, M. Bonfanti, C. Magagnotti & R. Fanelli

*Laboratory of Environmental Pharmacology and Toxicology,  
Istituto di Ricerche Farmacologiche 'Mario Negri', Via Eritrea 62  
20157 Milan, Italy*

The capacity of the isolated rat urinary bladder to metabolize chemical carcinogens was studied. Under our experimental conditions, the bladder carcinogen *N*-nitrosobutyl-(4-hydroxybutyl)amine (NBHBA) was oxidized to *N*-nitrosobutyl(3-carboxypropyl)amine (NBCPA). A time-dependent increase in the amount of NBCPA formed and a simultaneous disappearance of NBHBA indicated that the bladder can metabolize NBHBA to the metabolite considered to be responsible for tumour induction in the urinary bladder of laboratory animals. After 15, 30, 60 and 120 min, the percentages of NBCPA formed were 10%, 21%, 35% and 61%, respectively, and 59%, 49%, 36% and 25% of NBHBA remained unchanged. When *N*-nitrosodi-*n*-butylamine (NDBA) was introduced into the isolated urinary bladder and incubated for 120 min, its oxidized metabolites NBHBA and NBCPA were formed, in amounts of 0.13% and 0.06% of the substrate added.

The urinary bladder is the target for several carcinogens, most of which require metabolic activation to exert their effect, the liver being considered the primary site of activation; the activated carcinogen is then transported *via* the blood to the bladder where the carcinogenic effect is exerted (Kadlubar *et al.*, 1977). However, studies using microsomal preparations of bladder transitional-cell epithelium or cultured cells show that these *in-vitro* systems also activate various carcinogens (Autrup *et al.*, 1981; Vanderslice *et al.*, 1985). Thus, urinary bladder epithelial cells are also able to activate urinary bladder carcinogens.

We have set up an experimental model that closely reproduces the *in-vivo* situation to investigate the biotransformation of urinary bladder carcinogens in the isolated target organ. NBHBA was used to study the bladder's capacity to form the carcinogenic metabolite NBCPA, an oxidized product of NBHBA, which is the result of  $\omega$ -oxidation of NDBA. NBCPA is reportedly responsible for experimental bladder tumour induction in different animal species (Okada & Ishidate, 1977). The cytochrome P450-dependent  $\omega$ -oxidation of NDBA to NBHBA and further oxidation of the latter compound to NBCPA were also studied.

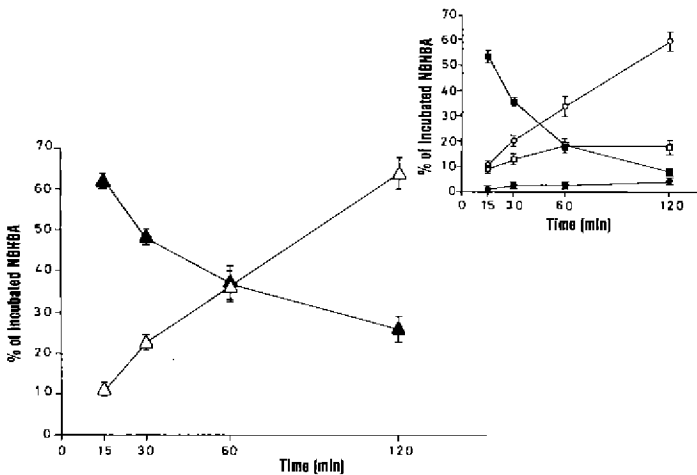
Ureters were ligated and cut and the urethra cannulated; the bladder was then removed, emptied, washed with saline and filled with 600  $\mu$ l rat urine, previously sterilized by filtration, containing 17 nmol NBHBA or 316 nmol NDBA. The urinary pH was found to be

7-7.5 and was not changed. The bladder was immersed in 5 ml 0.05 M phosphate buffer pH 7.4; bladders containing NMHBA were incubated for 15, 30, 60 and 120 min at 37°C, and those containing the substrate NDBA for 120 min. The reaction was terminated by emptying the bladders. Bladders, their contents and outside buffer were extracted at pH 4.5-5 with ethyl acetate; the extracts were evaporated to dryness and analysed for NBHBA and NBCPA content as their trimethylsilyl derivatives by gas chromatography-thermal energy analysis (GC-TEA), as described previously (Airoldi *et al.*, 1983b). When samples were also to be analysed for NDBA content, the volume of the ethyl acetate extracts was reduced to about 200  $\mu$ l before being evaporated to dryness and analysed for NBHBA and NBCPA.

Wishnok *et al.* (1982) showed that the urinary bladder is permeable to various nitrosamines. In concurrence with this finding, we observed in a preliminary experiment that certain amounts of the substrate and of the metabolite formed crossed the bladder wall and were found in the surrounding buffer. Thus, the disappearance of substrate and the formation of metabolite were always measured both inside and outside the bladder.

The formation of NBCPA from NBHBA incubated in isolated rat urinary bladders is reported in Figure 1. NBCPA formation was readily detectable, and 15 min after the start of the reaction its concentration was 1.85 nmol, accounting for about 10% of the substrate introduced into the bladder. NBCPA formation was linear up to 120 min; longer incubation times have not yet been investigated. As shown in Figure 1, most of the NBCPA formed was found in the buffer outside the bladder.

**Fig. 1.** Time-dependent formation of NBCPA and disappearance of NBHBA in the isolated rat urinary bladder incubated at 37°C after the introduction of 17 nmol NBHBA dissolved in 600  $\mu$ l urine



Values are percentages of the substrate added (mean  $\pm$  SD of four bladders).  $\Delta$ , NBCPA production (inside and outside);  $\nabla$ , NBHBA disappearance (inside and outside);  $\circ$ , NBCPA outside the bladder;  $\bullet$ , NBCPA inside the bladder;  $\square$ , NBHBA outside the bladder;  $\blacksquare$ , NBHBA inside the bladder

In order to verify whether some of the substrate added or metabolite formed was retained in bladder cells, some bladders were analysed for NBHBA and NBCPA content. The 60-min urinary bladder homogenates were found to contain  $1.47 \pm 0.83\%$  NBHBA and  $13.67 \pm 0.58\%$  NBCPA (mean  $\pm$  SD).

In a separate experiment, NBCPA (16 nmol) was incubated as described for 120 min in order to detect further NBCPA biotransformation. The GC-TEA chromatograms showed no peak other than NBCPA, indicating that metabolic pathways leading to compounds retaining the nitroso moiety are not of importance. The total amount of the compound recovered, both inside and outside the isolated urinary bladder, was  $95 \pm 4\%$  (mean  $\pm$  SD) of the NBCPA introduced into the bladder. Studies are in progress to assess whether the small percentage of unrecovered NBCPA is retained by the bladder cells or is  $\alpha$ -hydroxylated or metabolized through an unknown metabolic pathway.

When the NBHBA precursor NDBA was incubated in the isolated bladder,  $41.2 \pm 4.2\%$  remained unchanged (inside plus outside). The percentages of NBHBA and NBCPA produced from NDBA at 120 min were  $0.13 \pm 0.04\%$  and  $0.06 \pm 0.03\%$  (mean  $\pm$  SD).

The isolated rat bladder thus appears to be a simple tool for studying in-situ synthesis of carcinogens. An ideal experimental model should reproduce the in-vivo situation as closely as possible; it should be easy to handle; the carcinogen should be easily introduced and should be retained for a reasonable period of time. The experimental model described seems to meet these requirements and should be useful for various studies related to bladder carcinogenesis. It could be particularly suitable for testing the metabolic capacity of the urinary bladder, as well as for testing the direct effect of any particular compound.

Under the experimental conditions described, the enzymatic system considered — alcohol/aldehyde dehydrogenase and cytochrome P450-dependent monooxygenase — was present and functioning both in the urinary bladder and in the liver. Further studies are in progress to assess whether these metabolic transformations play a role in the induction of bladder tumours by NBHBA.

#### Acknowledgements

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## COULD *N*-NITROSAMINO PHOSPHATES BE TRANSPORT FORMS OF ACTIVATED *N*-NITROSAMINES ?

N. Frank, M. Wiessler, B.L. Pool & E. Frei

*Institute of Toxicology and Chemotherapy, German Cancer Research Center,  
Im Neuenheimer Feld 280, 6900 Heidelberg, Federal Republic of Germany*

**The stability of *N*-nitroso-*N*-methylamine-*N*-ethyl phosphate (NMEP) and *N*-nitroso-*N*-ethylamine-*N*-ethyl phosphate (NEEP) was studied in rat serum and in rat liver homogenate and found to be sufficiently high for the phosphates to be transported *in vivo*. In liver homogenates, the cleavage was more efficient, and a higher phosphatase activity was found in liver microsomal and cytosolic fractions than in serum. The substances exert a distinct activity in the *Salmonella typhimurium* mutagenicity test.**

Many *N*-nitrosodialkylamines that induce tumours in rat organs other than the liver are metabolized by liver microsomal monooxygenases. In a study by Druckrey *et al.* (1967), it was proposed that the mechanism of carcinogenesis in organs remote from the site of metabolic activation may be due to activation within the liver and subsequent transport of metabolites or conjugates to the target tissues. In our earlier studies, we demonstrated that the intermediates of dialkylnitrosamine metabolism, the hydroxy compounds and/or the diazohydroxides, are relatively stable in serum (Frank & Wiessler, 1983) and liver microsomal fractions (Appel *et al.*, 1981). A stable glucuronide of  $\alpha$ -hydroxylated *N*-nitroso-*tert*-butylmethylamine was also found in rat urine (Wiessler *et al.*, 1984).

Recently, we described a new class of *N*-nitrosamino compounds — *N*-nitrosamino  $\alpha$ -phosphate esters (NAP) (Frank & Wiessler, 1986b), which we showed were synthesized *via*  $\alpha$ -hydroxy transients. To investigate whether NAPs can be formed *in vivo* and can act as transport forms for activated nitrosamines, we studied their stability in serum and in liver homogenates. Furthermore, the alkaline phosphatase activity was measured in serum and in liver-cell cytosolic and microsomal fractions. Since conjugation of an active metabolite could also be a detoxifying step in the metabolism of nitrosamines, we investigated their mutagenic potency as a measure of biological activity.

### **Stability of nitrosamino phosphates in water**

The stability of NAPs in water depends on the pH value: NMEP and NEEP are stable in phosphate buffer at pH 8.5 and have a half-life of 22.0 h (NMEP) and 12.2 h (NEEP) at pH 7.0 (Frank & Wiessler, 1986b). The phosphates are thus much more stable than the corresponding acetylated *N*-nitrosamines (Edler *et al.*, 1983) or the  $\alpha$ -hydroxynitrosamines (Mochizuki *et al.*, 1980a).

### Stability of nitrosamino phosphates in serum

In order to elucidate the stability of NAPs in biological systems, they were incubated with rat serum or with rat serum diluted with phosphate buffer pH 8.5 at 37°C. At incremental times, the concentrations were measured by high-performance liquid chromatography (HPLC) after protein precipitation with methanol. (For HPLC conditions, see Frank & Wiessler, 1986b.) At a concentration of 8 nmol/mg protein, NMEP and NEEP were degraded, with half-lives of 1.0 h and 3.2 h, respectively. With a lower protein content (16 nmol/mg protein), the half-lives increased to 5.1 h for NMEP and 56.6 h for NEEP; at even lower protein concentrations (40 and 80 nmol NAP/mg protein), no degradation was observed. When the serum was denatured by heating, also no degradation occurred during 24 h. We conclude, therefore, that the degradation of NAPs is catalysed enzymatically, the catalysis being more effective for NMEP than for NEEP.

### Stability of nitrosamino phosphates in rat liver homogenate

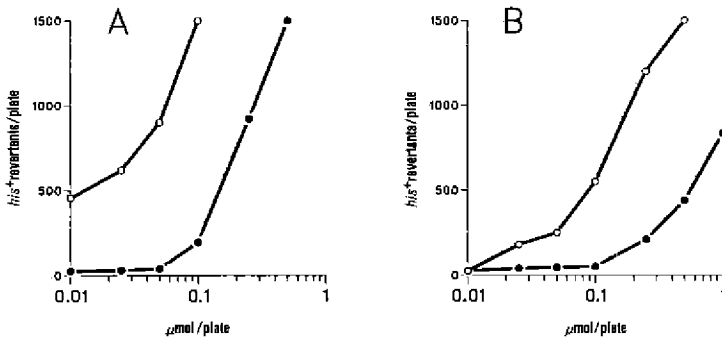
Rat liver was isolated and homogenized in nine volumes of 0.066 M sodium phosphate, dibasic pH 8.5. NMEP and NEEP were added at a concentration of 20 nmol/mg protein. At incremental times, aliquots were extracted, and the content of NAP was estimated by HPLC analysis. Half-lives of 5.9 h and 4.6 h were found for NMEP and NEEP, respectively. No degradation was seen in heat-denatured liver homogenate. With respect to protein content, the degradation of NAPs in liver homogenate is more effective than that in serum, but even the enzymatic degradation of NAPs is much slower than the hydrolysis of nitrosamino acetates or nitrosamino- $\alpha$ -hydroxides.

### Activity of alkaline phosphatase in rat serum and liver cytosolic and microsomal fractions

Since the NAPs are stable over hours in rat serum or rat liver homogenate, they are either transport forms which are split at the target site or are detoxification products which are eliminated. Therefore, the activity of alkaline phosphatase was measured in rat serum and in liver cytosolic and microsomal fractions. A classic photometric assay (Boehringer, Mannheim) was used to measure the increase of *para*-nitrophenol liberated from *para*-nitrophenylphosphate at 405 nm. The protein contents used were 1, 5 and 10 mg/ml. In serum and in cytosolic fractions, the reaction was linear, with a mean activity of  $13.8 \pm 1.2$  U/mg protein for serum and  $19.4 \pm 0.5$  U/mg for the cytosolic fraction. In the microsomal fraction, the enzyme reaction was nonlinear, with a mean activity of  $28 \pm 12.1$  U/mg protein. The high phosphatase activity in the microsomal fraction could be responsible for the more rapid degradation of NAPs in liver homogenate than in serum.

### Mutagenicity of NMEP and NEEP

NMEP and NEEP were tested for their mutagenicity in *Salmonella typhimurium* TA1535, according to the method of Pool and Wiessler (1981). The tests were performed with and without addition of alkaline phosphatase, which may cleave the NAPs to their reactive  $\alpha$ -hydroxy forms. The results are shown in Figure 1. Both compounds exert distinct mutagenic activity. Contrary to expectation, the presence of alkaline phosphatase diminished the mutagenic response of the compounds. The explanation may be that degradation of NAPs in the culture medium outside the bacteria lowers the amount of active substance entering bacteria to exert the mutagenic reaction.

**Fig. 1. Mutagenic activity of NMEP (A) and NEEP (B) in *S. typhimurium* TA1535**

Tests were performed in the presence of buffer pH 7.5 (empty symbols) and with added phosphatase (filled symbols).

In conclusion, the results show that NAPs are stable enough to be transported *in vivo*, but can be degraded by alkaline phosphatase. This may be decisive for their toxicity or elimination as detoxification products of  $\alpha$ -hydroxylated *N*-nitrosamines.



## CHEMICAL AND MUTAGENIC PROPERTIES OF $\alpha$ -PHOSPHONOOXYNITROSAMINES

M. Mochizuki<sup>1,2,3</sup>, A. Ikarashi<sup>1</sup>, A. Suzuki<sup>1</sup>, N. Sekiguchi<sup>1,2</sup>,  
T. Anjo<sup>2</sup> & M. Okada<sup>2</sup>

<sup>1</sup>Kyoritsu College of Pharmacy, Shibakoen 1-5-30, Minato-ku,  
Tokyo 105; and <sup>2</sup>Tokyo Biochemical Research Institute, Takada 3-41-8,  
Toshima-ku, Tokyo 171, Japan

The chemical and mutagenic properties of the products of solvolysis of  $\alpha$ -acetoxynitrosamines in phosphate buffer were investigated.  $\alpha$ -Acetoxynitrosamines decomposed in two ways: *O*-acyl fission yielded  $\alpha$ -hydroxynitrosamines, which decomposed into aldehydes and alcohols, while *O*-alkyl fission gave a resonance hybrid of  $\alpha$ -*N*-nitrosocarbenium and -iminium ions, which was trapped with phosphate and afforded  $\alpha$ -phosphonooxynitrosamine. Formation of  $\alpha$ -phosphonooxynitrosamines was dependent on the structure of  $\alpha$ -acetoxynitrosamines; those with a secondary  $\alpha$ -phosphonooxy group, including cyclic nitrosamines, were easily formed, while among those with a primary phosphonooxymethyl group, only those with an alkyl group containing a branched  $\alpha$ -carbon as isopropyl, *sec*-butyl and *tert*-butyl were isolated. They were good substrates of alkaline phosphatase and showed a nuclear magnetic resonance spectrum due to the presence of a phosphorus atom. They were decomposed by acid catalysis, and the rate was dependent on the structure. They were directly mutagenic in bacterial tester strains, except for a compound with a *tert*-butyl group. The activity was similar or stronger in *Salmonella typhimurium* TA1535 and much weaker in *Escherichia coli* WP2 and WP2 *hcr* than those of  $\alpha$ -acetoxynitrosamines. Stability in neutral aqueous solution and the strong mutagenicity of  $\alpha$ -phosphonooxynitrosamines suggested their possible involvement in metabolic activation as a precursor of  $\alpha$ -hydroxynitrosamines, and also in the organotropic carcinogenicity of *N*-nitrosodialkylamines as a transport form.

*N*-Nitroso iminium ion is possibly a precursor of  $\alpha$ -hydroxynitrosamines, the presumed active metabolite of carcinogenic and mutagenic *N*-nitrosodialkylamines, and was formed as a reactive intermediate in the hydrolysis of  $\alpha$ -acetoxynitrosamines, giving  $\alpha$ -phosphonooxynitrosamines by reaction with phosphate. This paper describes the chemical and mutagenic properties of  $\alpha$ -phosphonooxynitrosamines, which may be involved in the metabolic activation of *N*-nitrosodialkylamines to human carcinogens.

### Formation of $\alpha$ -phosphonooxynitrosamines

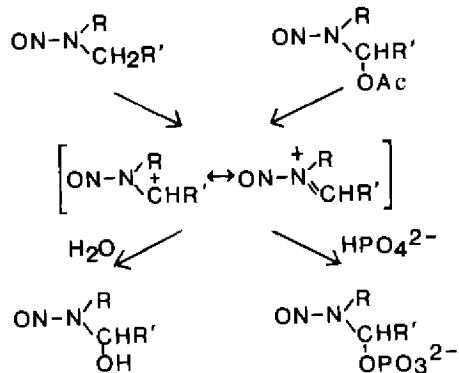
An anomalous behaviour was observed in the hydrolysis of  $\alpha$ -acetoxynitrosamines; some changed by solvolysis in phosphate buffer to new compounds which were mutagenic and retained ultra-violet absorption due to the N—NO group. The rate of decomposition of *N*-nitroso-*N*-(1-acetoxyalkyl)alkylamines in phosphate buffer of different pH was determined as a pseudo-first-order reaction from the decrease in their ultra-violet absorption, and was affected by both the pH of the buffer and the kind of alkyl group. Generally,

<sup>3</sup>To whom correspondence should be addressed

compounds with a primary acetoxy group (acetoxymethyl) were more stable than those with a secondary one, and acetoxymethylnitrosamines with a normal alkyl chain were more stable than those with a branched  $\alpha$ -carbon.

When  $\alpha$ -acetoxynitrosamines decompose through  $\alpha$ -hydroxynitrosamine, only alcohol and aldehyde are formed and no strong ultra-violet absorption is expected (Mochizuki *et al.*, 1980a). However, after the solvolysis of some  $\alpha$ -acetoxynitrosamines in phosphate buffer, ultra-violet absorption, with a maximum similar to those of  $\alpha$ -acetoxynitrosamines, remained after the reaction. The production of new compounds during hydrolysis in phosphate buffer was noted with secondary  $\alpha$ -acetoxynitrosamines (*1a-1d*),  $\alpha$ -acetoxycyclic nitrosamines (*1h, 1i*) and primary acetoxymethylnitrosamines, with a branched alkyl group as *tert*-butyl (*1e*), *sec*-butyl (*1f*) and isopropyl (*1g*) (Fig. 1, Table 1), but hardly at all with acetoxymethyl compounds with a normal alkyl chain. The ratio of the absorption remaining after the reaction to the original absorption of  $\alpha$ -acetoxynitrosamines was dependent on the pH of phosphate buffer, and was maximal in the pH range 7-9.

**Fig. 1. Possible involvement of *N*-nitrosoiminium and -carbonium ions in metabolic activation of *N*-nitrosodialkylamines and formation of  $\alpha$ -phosphoxynitrosamines from  $\alpha$ -acetoxynitrosamines**



resulted in an increase in the yields of new products, and a plot of reciprocal values of the absorption remaining after the reaction *versus* reciprocal values of the concentration of phosphate was linear. The linearity was explained by the mechanism shown in Figure 1. For example, the calculated value suggests that 65% of *1b* changed to *N*-nitrosoiminium intermediate, and 92% of the intermediate was trapped with phosphate. The yield expected by calculation (56%) was similar to the actual yield of isolation (56%), as described below.

The reaction was followed also by simultaneous changes in ultraviolet and high-performance liquid chromatography (HPLC) (LiChrosorb RP-18, CH<sub>3</sub>CN-phosphate buffer) spectra. As peak heights of  $\alpha$ -acetoxynitrosamine in HPLC decreased, those of a new peak increased. The rate constants measured from the changes in ultra-violet spectra were identical with those obtained from HPLC, and the rate of formation of the product was

The rate of hydrolysis was also determined in aqueous solutions other than phosphate buffer. Although the half-life of hydrolysis was similar, no strong ultra-violet absorption remained in borate or Tris buffers or in water. This suggests that the product with a chromophore was derived from the reaction of an intermediate with phosphate, and the rate was independent of the solutes used. Product formation did not affect the rate of solvolysis, indicating that the rate-determining step was decomposition of the  $\alpha$ -acetoxynitrosamines.

Since stable products were derived from phosphate and  $\alpha$ -acetoxynitrosamines, the effect of the concentration of phosphate was examined. The rate constant was independent of the concentration, which again suggested that the rate-determining step occurs prior to the reaction with phosphate. The absorption remaining after the reaction was dependent on the concentration of phosphate; an increase

**Table 1. Mutagenicity of  $\alpha$ -acetoxy- and  $\alpha$ -phosphonoxy nitrosamines in three microbial strains**

R ON-NCHR' X		Revertants/ $\mu$ mol							
		<i>S. typhimurium</i> TA1535		<i>E. coli</i> WP2		<i>E. coli</i> WP2 <i>hcr</i> <sup>-</sup>			
R	R'	1	3	1	3	1	3	1	3
a:	Me	Pr	119 200	255 000	200	2.0	130	2.0	
b:	Et	Me	6 000	6 100	1 600	22	950	29	
c:	Pr	Et	13 800	25 900	530	16	640	22	
d:	Bu	Pr	16 700	31 500	410	20	690	69	
e:	isoPr	H	84	150	3.4	0.6	1.9	0	
f:	<i>sec</i> -Bu	H	52	420	1.7	0	1.4	0	
g:	<i>tert</i> -Bu	H	0	0	0	0	0	0	
h:	-(CH <sub>2</sub> ) <sub>3</sub> -		11 200	11 000	540	7.5	2 700	20	
i:	-(CH <sub>2</sub> ) <sub>4</sub> -		1 700	2 600	48	8.5	460	30	

*1*, X = OAc; 3; X = OPO<sub>3</sub><sup>2-</sup>. Approximate values for the number of revertants induced per  $\mu$ mol of compound were calculated from the linear portion of the dose-response curve for each compound by the least-squares method.

also identical with the rate of decomposition of the  $\alpha$ -acetoxy nitrosamines. Thus, the rate of formation of the products was related not to the concentration of phosphate, but to the rate of decomposition of  $\alpha$ -acetoxy nitrosamines. This suggested that the reaction was an S<sub>N</sub>1 mechanism, with the rate-determining step the elimination of an  $\alpha$ -acetoxy group.

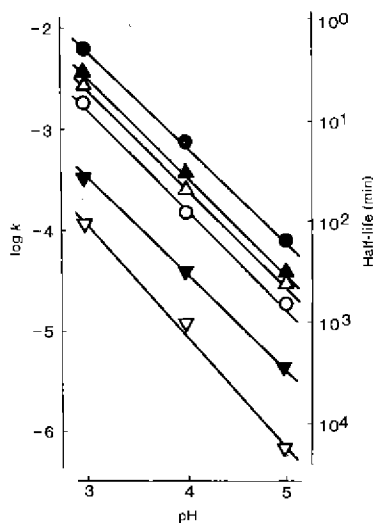
Decomposition of an  $\alpha$ -acetoxy nitrosamine (*1e*) and of the corresponding  $\alpha$ -hydroxy nitrosamine (*2e*) was compared. In *1e*, the ultra-violet absorption remaining after the reaction increased with phosphate concentration, whereas, in *2e*, neither the product nor the concentration of phosphate affected the ultra-violet absorption remaining after the reaction. Thus, in the case of  $\alpha$ -hydroxy nitrosamine in phosphate buffer, decomposition through iminium and carbonium ions is not a major pathway, but heterolysis, releasing aldehydes to alkyldiazohydroxide, is a major one.

### Chemical properties of $\alpha$ -phosphonoxy nitrosamines

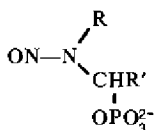
The reaction product was isolated by recrystallization as the sodium or cyclohexylammonium salt, and was characterized by its physico-chemical properties as  $\alpha$ -phosphonoxy nitrosamine (*3*). The resonance of protons in  $\alpha$ -methylene or methine was split by coupling with a phosphorus atom. The phosphonoxy nitrosamines were also good substrates for alkaline phosphatase. For example, a product of the solvolysis of *1b* in phosphate buffer is *N*-nitroso-*N*-(1-phosphonoxyethyl)ethylamine (*3b*), isolated as cyclohexylammonium salt in 56% yield. Frank and Wiessler (1986b) reported formation of this product recently. *3b* was stable in acetonitrile, ethanol and basic aqueous solution. Figure 2 shows that, in acidic solution,  $\alpha$ -phosphonoxy nitrosamines decompose by acid catalysis, with a linear relationship of the rate constant to the concentration of oxonium ion, and the rate is dependent on the alkyl group. The dependence of the rate constant

on the structure was similar to that for  $\alpha$ -acetoxynitrosamines. Faster decomposition was observed for those with a secondary  $\alpha$ -phosphonoxy group, and, among phosphonoxy methyl nitrosamines, those with a *tert*-butyl decomposed faster than those with a *sec*-butyl. The salt of **3b** was converted to an  $\alpha$ -methoxynitrosamine by treatment with methanol in the presence of an acid catalyst. This reaction suggested the involvement of *N*-nitrosoiminium ion derived from  $\alpha$ -phosphonoxy nitrosamine, as observed for  $\alpha$ -acetoxynitrosamines (Mochizuki *et al.*, 1980b).

**Fig. 2. Acid catalysis in the decomposition of  $\alpha$ -phosphonoxy nitrosamines in aqueous solution**



The rate constant,  $k$ , of decomposition in 0.2 M sodium phosphate solution with pH 3, 4 and 5 was calculated from the time-dependence of the decrease of the logarithmic value of ultra-violet absorption at 227 nm using the least-squares method. (R, R') = (Me, Pr):  $\circ$ , (Et, Me):  $\bullet$ , (Pr, Et):  $\Delta$ , (Bu, Pr):  $\blacktriangle$ , (*sec*-Bu, H):  $\nabla$ , (*tert*-Bu, H):  $\blacktriangledown$

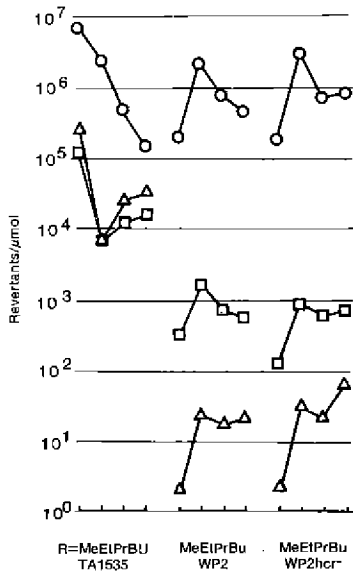


which, in turn, were about 100 times stronger than  $\alpha$ -phosphonoxy nitrosamines, although the effect of the alkyl group on the relative activity was similar in all three  $\alpha$ -oxygenated nitrosamines. In *S. typhimurium*, the effect of the alkyl group on relative mutagenicity was similar in  $\alpha$ -acetoxy- and in  $\alpha$ -phosphonoxy nitrosamines, but the mutagenicity had a

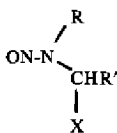
### Mutagenic properties of $\alpha$ -phosphonoxy nitrosamines

The mutagenicity of the products was assayed in *S. typhimurium* TA1535 and *E. coli* WP2 and WP2 *hcr*. Table I shows a comparison between the activity of the corresponding  $\alpha$ -phosphonoxy- and  $\alpha$ -acetoxynitrosamines. The activity of isolated  $\alpha$ -phosphonoxy nitrosamines accounted for the total mutagenic activity of the reaction mixture of  $\alpha$ -acetoxynitrosamines in phosphate buffer. They were directly mutagenic in all strains tested; the potency was increased in *S. typhimurium* and decreased in *E. coli* when compared to the activity of the corresponding  $\alpha$ -acetoxynitrosamines (Mochizuki *et al.*, 1979). Of the phosphonoxy methyl nitrosamines, compounds with a *tert*-butyl are not mutagenic, and those with a *sec*-butyl or isopropyl showed weaker mutagenicity, with a pattern similar to that of secondary phosphonoxy compounds: stronger in *S. typhimurium* and weaker in *E. coli* than the corresponding acetoxymethyl compounds. Figure 3 shows a comparison of the patterns of mutagenicity of  $\alpha$ -phosphonoxy nitrosamines with those of the corresponding  $\alpha$ -hydroperoxy- and  $\alpha$ -acetoxynitrosamines in three different microbial strains, with respect to the effect of the alkyl group. The chemical and mutagenic properties of hydroperoxymethyl nitrosamines proved to be similar to those of hydroxymethyl nitrosamines (Okada *et al.*, 1980; Mochizuki *et al.*, 1982). In the *E. coli* strains,  $\alpha$ -hydroxyperoxy nitrosamines showed about 1000 times more activity than the  $\alpha$ -acetoxynitrosamines,

**Fig. 3. Comparative mutagenic activity of  $\alpha$ -hydroperoxy-,  $\alpha$ -acetoxy- and  $\alpha$ -phosphonoxy nitrosamines in *S. typhimurium* TA1535, *E. coli* WP2 and *E. coli* WP2 *hcr*<sup>-</sup> with regard to the effect of the alkyl group**



The specific activity was calculated from the slopes of the linear dose-response curve in the concentration range before the toxic effect appeared. Activity per  $\mu\text{mol}$  of compounds was plotted against alkyl group of alkylating species, methyl, ethyl, propyl and butyl.



X = OOH:  $\circ$ ,  
 = OAc:  $\square$ ,  
 =  $\text{OPO}_3^-$ :  $\Delta$ .

R	R'
Me: Me, Pr	
Et: Et, Me	
Pr: Pr, Et	
Bu: Bu, Pr	

different pattern and was much weaker than that of the corresponding  $\alpha$ -hydroperoxynitrosamines, which showed similar activity in the three bacterial tester strains.  $\alpha$ -Acetoxynitrosamines were more mutagenic in *S. typhimurium* strains than in *E. coli* strains (Mochizuki *et al.*, 1979), unlike  $\alpha$ -hydroxy-,  $\alpha$ -hydroperoxy- and  $\alpha$ -oxonitrosamines (Okada *et al.*, 1980), which can be partly explained by the intermediary formation of a product like  $\alpha$ -phosphonoxy nitrosamines.

A possible involvement of carbonium and iminium ions in the metabolic activation of carcinogenic *N*-nitrosodialkylamines as a precursor of  $\alpha$ -hydroxynitrosamines, and a role of  $\alpha$ -phosphonoxy nitrosamines in the organ-specific carcinogenesis of *N*-nitrosodialkylamines as a transport form may be important in the mechanism of human carcinogenesis and are a subject of research in progress.

**Acknowledgement**

This work was supported in part by the Grants-in-Aid for Cancer Research from the Ministry of Health and Welfare, and the Ministry of Education, Science and Culture.

## $\alpha$ -GLUCURONIDES OF *N*-NITROSOMETHYLBENZYLAMINE

M. Wiessler & G. Rosnagel

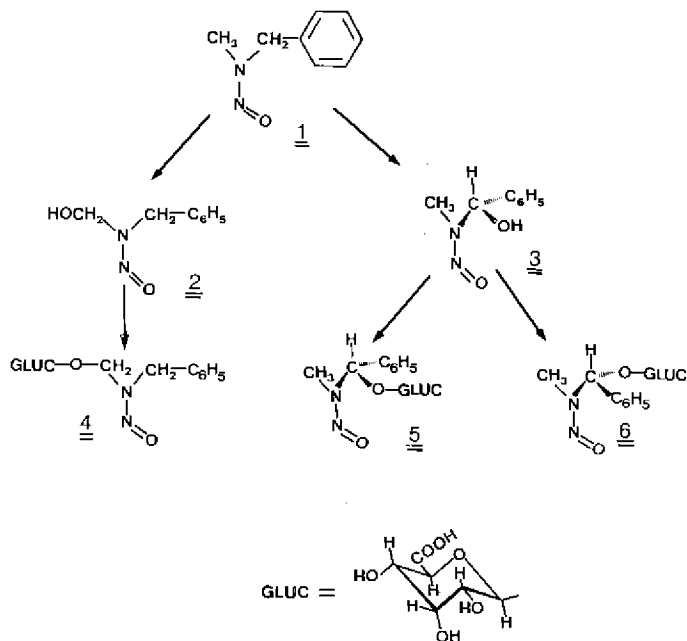
*German Cancer Research Center, Im Neuenheimer Feld 280,  
D-6900 Heidelberg, Federal Republic of Germany*

After administration of *N*-nitroso[<sup>14</sup>C-methyl]benzylamine to rats, a glucuronide was demonstrated in the urine, the structure of which could be assigned on the basis of synthetic marker compounds. These findings offer an explanation for the failure to detect benzylation of DNA after administration of *N*-nitrosomethylbenzylamine (NMBzA). The glucuronides were synthesized by a combination of enzymatic and chemical reactions.

Formation of the  $\alpha$ -glucuronide of *N*-nitroso-*tert*-butylmethylamine *in vivo* was described recently (Wiessler *et al.*, 1984), indicating that the intermediate  $\alpha$ -hydroxy compound is stable enough to be a substrate for UDP-glucuronyltransferase. Since *N*-nitroso-*tert*-butylmethylamine is not carcinogenic in rats (Gold *et al.*, 1981), formation of this glucuronide cannot be related to the carcinogenicity of nitrosamines.

NMBzA induces oesophageal tumours by any route of application. By the two routes of  $\alpha$ -hydroxylation, leading to 2 and 3 (Fig. 1), two isomeric  $\alpha$ -glucuronides can be formed. Since the  $\alpha$ -C atom bearing the hydroxy group in 3 represents a chiral centre, two diastereomeric glucuronides, 5 and 6, can be derived from 3. After administration of NMBzA *in vivo*, three glucuronides are excreted in the urine. In order to assign correct structures to these glucuronides, they were synthesized.

The synthetic material obtained by the published chemical procedure (Wiessler & Braun, 1980) represents a mixture of  $\alpha$  and  $\beta$  glucuronides, which could not be separated by any analytical technique available to us. Therefore, a combination of chemical and biochemical methods was developed to synthesize glucuronides 4, 5 and 6. The  $\alpha,\beta$ -glucoside mixture was deprotected by ammonia in methanol. After lyophilization, the  $\alpha,\beta$  mixture was treated with  $\alpha$ -glucosidase. Completeness of the scission of the  $\alpha$ -glucoside was tested by subsequent addition of  $\beta$ -glucosidase, which resulted in complete disappearance of the glucoside peak in high-performance liquid chromatography (HPLC). Oxidation of the glucoside with oxygen, according to the method of Heyns and Beck (1957), with minor modifications, was monitored by HPLC. Whereas 4 was rather stable under these conditions, formation of the diastereomers 5 and 6 was accompanied by formation of benzoic acid, indicating lower hydrolytic stabilities of 5 and 6. Purification was achieved by medium-pressure liquid chromatography (MPLC), and the glucuronic acids were isolated as their ammonium salts in crystalline form. Proton nuclear magnetic resonance spectra and elemental analysis were in accordance with the proposed structures. Complete disappearance of ultra-violet absorption at 235 nm was observed after the addition of  $\beta$ -glucuronidase at pH 7.4. The absolute stereochemistry of 5 and 6 has not yet been assigned. Recently, one diastereomer was isolated, and structural assignment will be done by nuclear magnetic resonance spectroscopy.

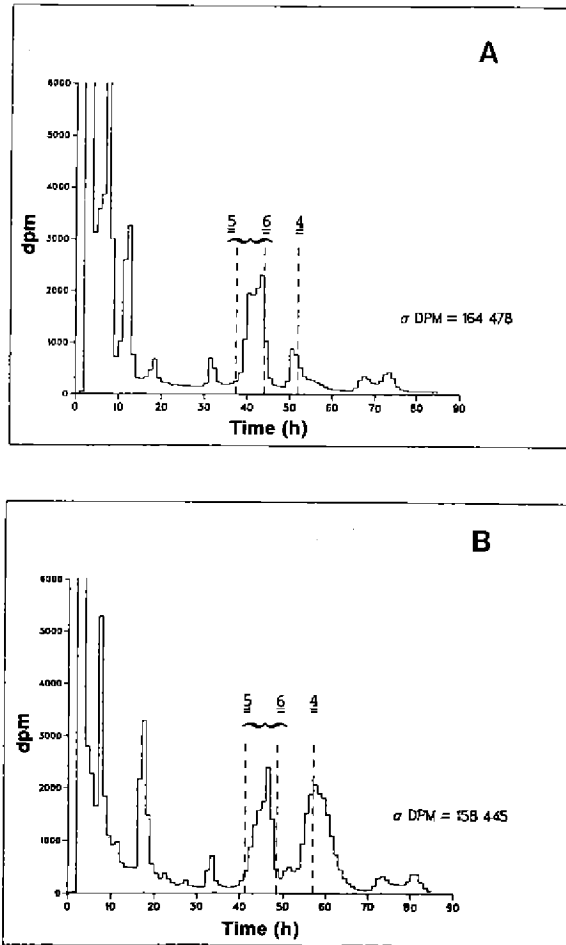
**Fig. 1.** Metabolic activation of NMBzA (*1*) and glucuronides that can be formed

$^{14}\text{C}$ -Methyl-labelled NMBzA was synthesized on the basis of  $\text{K}^{14}\text{CN}$  with a specific activity of 25 mCi/mmol. Two male Sprague-Dawley rats received 4.2 mg/kg  $^{14}\text{C}$ -NMBzA subcutaneously and were housed together in a metabolic cage. Over 24 h, 32% of the total radioactivity was exhaled as carbon dioxide and 8.4% was excreted in the urine. The urine was concentrated to 2 ml by lyophilization and the proteins removed by addition of ethanol and filtration.

After lyophilization, the residue was dissolved in a minimum amount of water and transferred to a RP18 column (30 × 2 cm) and eluted with 1 M sodium phosphate, pH 7.6 with 10% methanol under MPLC conditions (9.5 ml/min). After a run of 60 ml, 100 ml of eluent containing the glucuronides was collected, the solvents evaporated and the residue dissolved in 2 ml water; and 100- $\mu\text{l}$  samples were separated on HPLC after the addition of marker compounds. In all, 84 fractions were collected and  $^{14}\text{C}$  radioactivity measured by liquid scintillation counting.

In one of these 100- $\mu\text{l}$  samples, 23 mg saccharolacton was dissolved before the addition of 20  $\mu\text{l}$   $\beta$ -glucuronidase (in glycol, Böhringer). The samples were analysed as described above and the results are shown in Figure 2. These findings clearly demonstrate that the glucuronide **4** is excreted in urine; glucuronides **5** and **6** are chemically less stable than **4** and are present only in minor amounts. Glucuronide formation may thus be a detoxification pathway for the benzylating agent **3**, inhibiting benzylation of DNA (Hodgson *et al.*, 1982) by NMBzA.

**Fig. 2. High-performance liquid chromatography (HPLC) chromatogram of 100- $\mu$ l samples incubated with  $\beta$ -glucuronidase (A) and  $\beta$ -glucuronidase and saccharalactone (B)**



The dotted lines indicate the retention times of the synthetic standards 4, 5 and 6 (Fig. 1). HPLC conditions: Shandon ODS Hypersil 120  $\times$  8 mm; eluent: 2 mM tetrapentylammonium-bromide, 0.5 mM boric acid pH 7.6 in water, 5% thioformamide, 10% methanol, at a rate of 2 ml/min. The shift in retention times is caused by a 40  $\times$  8 mm precolumn filled with the same material.



## EFFECT OF ASCORBIC ACID ON THE METABOLISM OF N-NITROSOPIPERIDINE

M. Nakamura<sup>1</sup>, Y. Osada<sup>1</sup>, Y. Horiguchi<sup>1</sup>, T. Nakaoka<sup>1</sup>, K. Ito<sup>1</sup>  
& T. Kawabata<sup>2</sup>

<sup>1</sup>Kanagawa Prefectural Public Health Laboratory, 52-2 Nakao-cho, Asahi-ku, Yokohama 241; and <sup>2</sup>National Institute of Health, Department of Biomedical Research on Food, 2-10-35 Kamiosaki, Shinagawa-ku, Tokyo 141, Japan

Guinea-pigs were fed diets containing 48, 200 or 2000 mg/kg ascorbic acid (AsA) for 20-30 days and given a single oral dose of 50 mg *N*-nitrosopiperidine (NPIP)/kg body weight. The levels of glutaric acid, 3-hydroxy-NPIP and 4-hydroxy-NPIP, but not of 1,5-pentanediol, in urine increased with the dose of AsA in the diet.

The effect of AsA on tumour incidence is controversial: some authors report that it inhibits cancer formation (Pipkin *et al.*, 1969; Reddy *et al.*, 1982), others that it enhances it (Mirvish *et al.*, 1975; Fukushima *et al.*, 1982) and still others that it has no observable effect (Jones *et al.*, 1981). These discrepancies may be due to variations in experimental conditions or evaluative methods. Information on the effects of AsA on carcinogen metabolism is needed.

This study was intended to elucidate the effect of NPIP in guinea-pigs given an exogenous source of AsA, as well as its effect in humans. AsA was fed in the diet to guinea-pigs (Hartley, male, 24 weeks old) for 20-30 days at levels of 48, 200 and 2000 mg/kg. After a single oral dose of NPIP in olive oil, corresponding to one-quarter of the LD<sub>50</sub> (200 mg/kg orally in rats; Druckrey *et al.*, 1967), urine metabolites were determined at 24-h intervals.

### Isolation and identification of metabolites

A slight modification of the method of Singer *et al.* (1981) was used to isolate the metabolites. For 1,5-pentanediol and 3- and 4-hydroxy-NPIP, the method was as follows: urine (50 ml) was adjusted to pH 7.5, extracted with ethyl acetate in the presence of sodium chloride, concentrated to ~ 30 ml, applied to a Florisil column (1 cm i.d. × 10 cm) and concentrated under vacuum to 1.0 ml for gas chromatography-mass spectrometry (GC-MS). For glutaric acid, the aqueous layer of the urine phase was extracted with ethyl acetate at pH 7.5, adjusted to pH 1.5 and re-extracted with ethyl acetate; the organic extract was evaporated to dryness under vacuum. Diazomethane ether solution was then added, the solution stood for 1 h and was then extracted with cyclohexane, after adding 20 ml of water. The cyclohexane layer was applied to the Florisil column, eluted with 30 ml ethyl acetate and concentrated to 1.0 ml for GC-MS. Percentage recoveries of these substances in urine were as follows: 1,5-pentane-diol, 78.1 (± 3.9); glutaric acid, 47.4 (± 3.7); 3-hydroxy-NPIP, 84.8 (± 8.2); 4-hydroxy-NPIP, 83.8 (± 4); and NPIP, 85.8 (± 7.4).

3-Hydroxy-NPIP, 4-hydroxy-NPIP and NPIP were quantified by GC-MS using the single-ion monitoring method (electron ionization). 1,5-Pentanediol and dimethylglutarate could not be detected as the molecular ion, so these substances were determined by chemical ionization GC-MS, as above, with isobutane gas.

#### Effect of AsA on metabolites of NPIP in urine

The results are shown in Table 1. Excretion of urinary metabolites of NPIP, glutaric acid and 3- and 4-hydroxy-NPIP apparently increased with increasing doses of AsA in the diet. No 1,5-pentanediol was found. The enhanced rates of glutaric acid formation with higher doses of AsA in the diet were probably the result of an increase in cytochrome P450 levels in liver microsomes (Kuenzig *et al.*, 1977; Omaye *et al.*, 1979). As we reported previously, formation of 5-hydroxypentanal from NPIP incubated with liver microsomes *in vitro* was higher when animals were fed small amounts (200 mg/kg) of AsA in the diet as compared with those fed a higher dose (1000 mg/kg) (Nakamura *et al.*, 1985). This fact and the data in Table 1 suggest that AsA-induced guinea-pig liver microsomal cytochrome P450 is not directly associated with  $\alpha$ -hydroxylation of NPIP, while protein and nucleic acid modification by carbonium ion derived from  $\alpha$ -hydroxylation is accelerated in the liver. Furthermore, unmetabolized NPIP in urine remains at an almost constant level; the formation of 3- and 4-hydroxy-NPIP induced by ingestion of large amounts of AsA may suggest that  $\beta$ - and  $\gamma$ -hydroxylation are a substantial metabolic pathway of NPIP in guinea-pigs fed large amounts of AsA.

**Table 1. Yields of urinary metabolites in guinea-pigs fed varying concentration of AsA in the diet after administration of a single dose of NPIP<sup>a</sup>**

AsA in diet (mg/kg)	Glutaric acid	3-OH-NPIP	4-OH-NPIP	NPIP
48	ND	0.01	0.05	0.01
	ND	0.01	0.04	Trace
	ND	ND	0.01	0.01
200	0.04	0.13	0.36	0.01
	0.02	0.13	0.29	0.01
	0.02	ND	0.07	ND
2000	0.08	0.27	0.66	0.03
	0.05	0.22	0.52	0.03
	0.03	0.05	0.14	Trace

<sup>a</sup>% of original amount of NPIP in 48 h; ND, not detected; upper figures, urine collected up to 24 h; lower figures, urine collected over following 24 h

# EFFECT OF (+)-CATECHIN, DIMETHYL SULFOXIDE AND ETHANOL ON THE MICROSOME-MEDIATED METABOLISM OF TWO HEPATOCARCINOGENS, *N*-NITROSODIMETHYLAMINE AND AFLATOXIN B<sub>1</sub>

H.R. Prasanna, P.D. Lotlikar, N. Hacobian, L.L. Ho & P.N. Magee

*Fels Research Institute and Department of Pathology,  
Temple University School of Medicine, Philadelphia, PA 19140, USA*

Effects of catechin, a plant phenolic flavonoid, and of the commonly used organic solvents dimethyl sulfoxide (DMSO) and ethanol (EtOH) on the microsome-mediated metabolism of two hepatocarcinogens, *N*-nitrosodimethylamine (NDMA) and aflatoxin B<sub>1</sub> (AFB<sub>1</sub>), are presented. Using hamster liver microsomes as a source of mixed-function oxidases, it was shown that catechin at 0.1-0.2 mM levels had no effect on the oxidation of either carcinogen. However, at 1-5 mM levels it caused a concentration-dependent inhibition (38-70%) of the formation of formaldehyde from NDMA, and at the 5 mM level it caused a 40% inhibition of AFB<sub>1</sub>-DNA binding. DMSO and EtOH totally inhibited NDMA demethylase activity but had little effect on the binding of AFB<sub>1</sub> to DNA. These observations indicate that the mixed-function oxidases (cytochrome P450) essential for the metabolic activation of these carcinogens exhibit different sensitivities to different inhibitors.

NDMA and AFB<sub>1</sub> have been investigated extensively during the past decade and shown to be carcinogenic to a number of species (Newberne & Butler, 1969; Montesano & Magee, 1974; Magee *et al.*, 1974; Preussmann & Stewart, 1984). Since environmental exposure to these compounds through a variety of sources is highly possible (Shank *et al.*, 1972; Magee *et al.*, 1974), they pose a high risk to humans. NDMA and AFB<sub>1</sub> require cytochrome P450-dependent metabolic activation for expression of their carcinogenicity in several species (Magee *et al.*, 1974; Miller & Miller, 1977; Preussmann & Stewart, 1984). Scientists in many laboratories have searched for agents that inhibit their carcinogenic effects. Since several plant flavonoids, particularly catechin (Steele *et al.*, 1985), inhibit the metabolic activation of several polycyclic hydrocarbons, they are potential anticarcinogens. We report here the effects of catechin, DMSO and EtOH on microsomal oxidation of NDMA and AFB<sub>1</sub>, two hepatocarcinogens with different structures.

## **In-vitro metabolism of NDMA and AFB<sub>1</sub>**

Microsomal fractions from the livers of hamsters were prepared as described previously (Lotlikar *et al.*, 1980). The cytochrome P450 and protein contents of the microsomal fractions were estimated by the methods of Omura and Sato (1964) and Lowry *et al.* (1951), respectively. Catechin, EtOH and DMSO were all made up in phosphate buffer, pH 7.4, which remained at the same pH when the compounds were dissolved.

The incubation medium contained 100 mM phosphate buffer pH 7.4, 2 mM NADPH, 2  $\mu$ M  $^{14}$ C-NDMA or  $^3$ H-AFB<sub>1</sub>, 0.5-1 nmol cytochrome P450 or 1 mg microsomal protein, with or without various amounts of the test compounds. Samples incubated with AFB<sub>1</sub> contained 1 mg calf thymus DNA. All incubations were carried out at 37°C for 30 min. Formaldehyde generated from the samples incubated with NDMA was estimated as described previously (Prasanna *et al.*, 1985). DNA was isolated (Wang & Cerutti, 1980) from the samples incubated with AFB<sub>1</sub>, and the AFB<sub>1</sub>-DNA complex was quantified (Lotlikar *et al.*, 1984).

### Effects of EtOH and DMSO on the microsomal metabolism of NDMA

Results presented in Table 1 and Figure 1 show that the mixed-function oxidase (cytochrome P450) system that mediates the metabolism of NDMA is very sensitive to EtOH and DMSO, in contrast to the metabolism of AFB<sub>1</sub>. Since these experiments were performed with low concentrations of the carcinogens (2  $\mu$ M) and various concentrations of EtOH and DMSO (3-300 mM), the concentration-dependent inhibition is an effect on the pathophysiologically important NDMA demethylase (Pegg, 1980), as this is the enzyme operative at low substrate concentrations. Additionally, since formation of formaldehyde and alkylation of DNA by NDMA proceed in parallel (Jensen *et al.*, 1981), the present data suggest that EtOH might reduce the amount of DNA alkylation by NDMA. Other studies have shown that EtOH can act either as an inducer (Peng *et al.*, 1982) or an inhibitor of NDMA metabolism (Swann, 1982; Tomera *et al.*, 1984). Interestingly, addition of EtOH to microsomes *in vitro* inhibits NDMA activation (Schwartz *et al.*, 1980).

**Table 1. Effect of EtOH on microsomal metabolism<sup>a</sup> of NDMA and AFB<sub>1</sub><sup>b</sup>**

EtOH (mM)	NDMA		AFB <sub>1</sub>	
	Formaldehyde formed (pmol)	% of control	AFB <sub>1</sub> -DNA binding (pmol)	% of control
None	357	100	300	100
30	9	2.5	320	107
150	6	1.7	300	100
300	5	1.4	280	93

<sup>a</sup>Metabolism of NDMA measured by formaldehyde production and of AFB<sub>1</sub> by binding to calf thymus DNA

<sup>b</sup>Values are mean of triplicates; variation among triplicates was < 5%.

<sup>c</sup>2  $\mu$ M in 300 mM dimethyl sulfoxide

sensitive to the inhibitory effect of this phenol than that which mediates the oxidation of AFB<sub>1</sub> (70% versus 45% inhibition). The mechanism by which catechin exerts its effect on cytochrome P450 and/or NADPH-cytochrome c reductase needs further investigation. These preliminary data suggest, however, a different inhibition of the metabolism of these two carcinogens at the cytochrome P450 level, and studies of inhibitors on alkylation of DNA *in vivo* and on the carcinogenesis by NDMA and AFB<sub>1</sub> would be of interest.

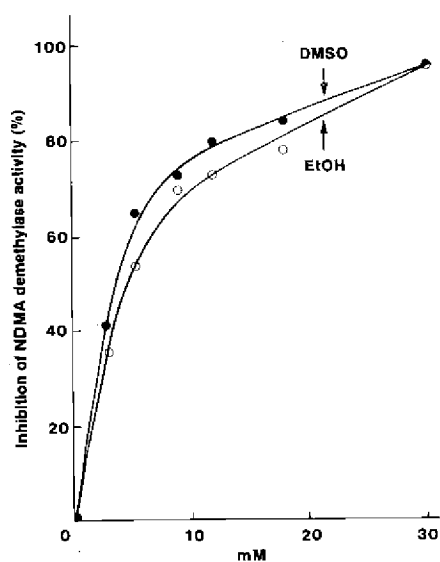
These findings are the first demonstration of the inhibitory effect of DMSO on the hepatic metabolism of NDMA, which had been predicted by others (Sosonowski *et al.*, 1976; Mori *et al.*, 1985).

### Effect of (+)-catechin on the *in-vitro* metabolism of NDMA

Results presented in Table 2 show that (+)-catechin inhibits the biotransformation of both NDMA and AFB<sub>1</sub> by microsomes. With high levels of catechin (0.2-5 M), the mixed-function oxidase that mediates the oxidative demethylation of NDMA is much more

**Table 2. Effect of (+)-catechin on the microsomal metabolism of NDMA and AFB<sub>1</sub><sup>a</sup>**

Catechin (mM)	NDMA		AFB <sub>1</sub>	
	Formaldehyde formed (pmol)	Inhibition (%)	AFB <sub>1</sub> -DNA binding (pmol)	Inhibition (%)
None	357	0	300	0
100	358	0	ND <sup>b</sup>	-
200	351	2	270	10
1000	222	38	240	20
2000	156	54	ND	-
5000	105	70	165	45

<sup>a</sup>Conditions as described in the footnotes to Table 1<sup>b</sup>ND, not determined**Fig. 1. Inhibition of NDMA demethylase activity by EtOH and DMSO**

Hamster liver microsomes were incubated with <sup>14</sup>C-NDMA, with various amounts of EtOH and DMSO, as indicated. NDMA demethylase activity was estimated by measuring the amount of formaldehyde formed. Each point represents the mean of triplicates for each concentration of inhibitor; variation among triplicates was < 5%.

#### Acknowledgements

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# EFFECT OF DIETARY SELENIUM ON BIOTRANSFORMATION AND EXCRETION OF MUTAGENIC METABOLITES OF *N*-NITROSODIMETHYLAMINE AND 1,1-DIMETHYLHYDRAZINE IN THE LIVER PERFUSION/CELL CULTURE SYSTEM

B. Beije, U. Olsson & A. Önfelt

*Department of Genetic and Cellular Toxicology,  
Wallenberg Laboratory, Stockholm University,  
S - 106 91 Stockholm, Sweden*

The mutagenicity of *N*-nitrosodimethylamine (NDMA) and 1,1-dimethylhydrazine (UDMH) has been studied in an isolated liver perfusion/cell culture system. The liver donors, male Wistar rats, were either selenium (Se)-deficient or had a physiologically adequate Se status (Se-supplemented). Mutagenicity was measured in perfusate and bile with Chinese hamster V79 cells as the genetic target. Se deficiency increased the mutagenic effect of NDMA in the perfusate, whereas no mutagenicity was detected in the bile of either Se-deficient or Se-supplemented livers. No significant increase in the mutagenicity of UDMH was seen in the perfusate with Se deficiency, but the bile became mutagenic. Se deficiency thus increased the mutagenicity of both NDMA and UDMH: with NDMA, the effect was observed in the perfusate, and with UDMH, in the bile.

The effect of dietary Se deficiency on the mutagenicity of NDMA and one of its proposed metabolites, UDMH (Daugherty *et al.*, 1977; Grilli & Prodi, 1975), has been investigated in an isolated liver perfusion/cell culture system (Beije *et al.*, 1979). Our interest in the effects of Se and NDMA emanates from the fact that (1) Sweden is a Se-deficient region, (2) the importance of dietary Se intake on cancer incidence has been indicated by several investigations (Combs & Clark, 1985) and (3) it has been established that humans are exposed to NDMA (Choi, 1985).

## Alteration in mutagenicity caused by Se deficiency

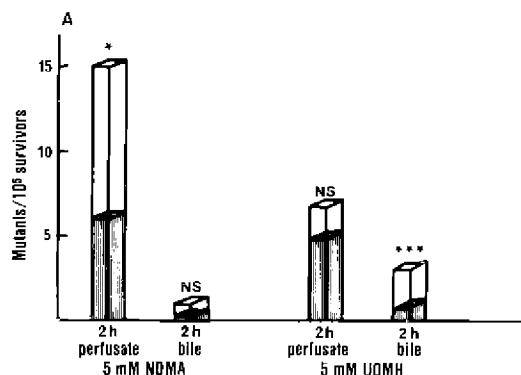
The isolated liver perfusion/cell culture system used was described in detail earlier (Beije *et al.*, 1979). Briefly, the isolated liver is perfused in a recirculating system which allows continuous exposure of V79 cells to potential mutagenic/carcinogenic metabolites released into the perfusate. The bile is collected separately, and samples tested for mutagenicity by incubation with V79 cells.

Adult male Wistar rats were used as liver donors, after six weeks on a Se-deficient diet with or without Se supplementation (0.2 ppm) in the drinking-water (Olsson *et al.*, 1984). Thus, the Se-supplemented rats were kept at a physiologically adequate Se level.

Se deficiency increased the mutagenic effect of both NDMA and UDMH (Fig. 1A), as well as NDMA-demethylase activity (Fig. 1B). The mutagenic activity was however, affected differently by the two compounds. A two-fold increase in mutagenicity was

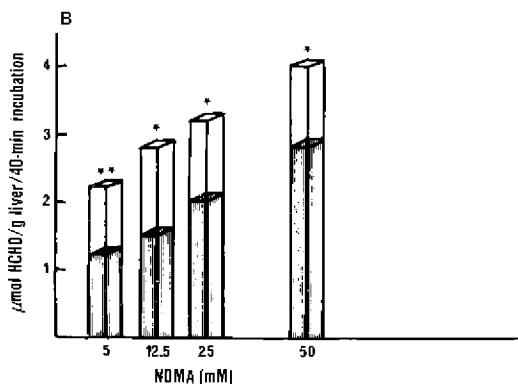
observed in the perfusate during metabolism of NDMA by Se-deficient livers compared to Se-supplemented livers, whereas no mutagenicity was observed in the bile. The concomitant increase in NDMA demethylase activity in the Se-deficient livers is the most likely reason for the observed increase in NDMA mutagenicity. During perfusion with UDMH, however, Se-deficient livers produced mutagenic bile, while the mutagenicity in the perfusate did not differ significantly from that of Se-supplemented livers. Thus, the dietary Se level may influence the biotransformation of promutagens and affect the excretion of mutagenic bile, which may have some relevance for gut carcinogenicity.

**Fig. 1A. Mutagenic response in V79 cells exposed to perfusate or bile in the isolated liver perfusion system, using Se-deficient (open columns) and Se-supplemented livers (filled columns)**



A. Each column represents the mean of six (NDMA) or three experiments (UDMH). The means  $\pm$  SE for Se-deficient livers were:  $14.9 \pm 4.3$  (NDMA, perfusate) and  $0.42 \pm 0.36$  (NDMA, bile),  $6.78 \pm 1.98$  (UDMH, perfusate) and  $3.04 \pm 0.81$  (UDMH, bile). The means  $\pm$  SE for Se-supplemented livers were:  $5.9 \pm 1.4$  (NDMA, perfusate) and  $0.06 \pm 0.05$  (NDMA, bile),  $4.76 \pm 0.65$  (UDMH, perfusate) and  $0.43 \pm 0.12$  (UDMH, bile). The initial concentration of each compound in the perfusate is indicated. Significance versus Se-supplemented: NS, not significant; \*,  $0.05 > p > 0.01$ ; \*\*\*,  $p < 0.005$ . Spontaneous mutation frequency was  $0.56 \pm 0.15$ , with no significant, direct effect of NDMA or UDMH. Both compounds caused a significant mutagenic effect in the perfusate of Se-supplemented livers compared to the direct effect ( $p < 0.005$ ). For further details, see Olsson *et al.* (1984) and Beije *et al.* 1984).

**Fig. 1B. NDMA-demethylase activity in hepatic postmitochondrial supernatant incubated with NDMA at the concentrations indicated.**



B. Significance, based on combined probability: Se-deficient (open columns) versus Se-supplemented (filled columns): \*,  $0.05 > p > 0.01$ ; \*\*,  $p < 0.01$ . For details, see Olsson *et al.* (1984).

### Relevance to humans

It is well established that the Se level varies greatly between geographically different regions. Depending on the source of food and eating habits, it can be assumed that large groups of people may have suboptimal Se intakes. Heavy alcohol consumption may also

lead to a reduced Se level (Dworkin *et al.*, 1984). Furthermore, it has been indicated in epidemiological studies that low Se status is associated with increased cancer risk (Combs & Clark, 1985). Finally, humans are also exposed to a large number of nitroso compounds through food, tobacco products and alcoholic beverages as well as endogenously produced nitrosamines (Choi, 1985).

Both epidemiological studies and studies with experimental animals suggest mechanisms by which Se may function as a risk modifier in carcinogenesis. The present results with dietary Se deficiency and mutagenicity of NDMA and UDMH give further support to this assumption. The liver is an important organ for the metabolism of several nitrosamines. To obtain a better understanding of the potential hazard to humans who have suboptimal Se levels and who are exposed to nitrosamines, it is essential to continue studying the interaction between these factors in experimental systems which allow manipulation of the various factors involved.

#### **Acknowledgement**

The present work was supported by the National Swedish Environmental Protection Board, Swedish Council for Planning and Coordination of Research and the Swedish Natural Science Research Council.



## DIFFERENT EFFECTS OF CHEMICALS ON METABOLISM OF *N*-NITROSAMINES IN RAT LIVER

T. Kawanishi, Y. Ohno, A. Takahashi, A. Takanaka & Y. Omori

*Division of Pharmacology, Biological Safety Research Center,  
National Institute of Hygienic Sciences, 1-18-1, Kamiyoga,  
Setagaya-ku, Tokyo 158, Japan*

The effects of phenobarbital (PB), 3-methylcholanthrene (MC), pyrazole (PY) and ethanol (EtOH) pretreatment on *N*-nitrosodimethylamine (NDMA), *N*-nitrosobutylmethylamine (NBMA) and *N*-nitrosomethylbenzylamine (NMBzA) metabolism were examined in rats. In isolated hepatocytes, PB increased the metabolic decomposition of NBMA and NMBzA, and MC increased that of NBMA; PY and EtOH increased only that of NDMA. In studies of hepatic microsomal dealkylation, PB increased NBMA debutylation and NMBzA debenzylation, and MC increased NBMA debutylation; PY and EtOH increased NDMA demethylation selectively. Several cytochrome P450 (P450) species were active in dealkylating nitrosamines, indicating that the organ-specific carcinogenicity of nitrosamines might be changed by various P450 inducers.

The enzymology of nitrosamine metabolism is not clearly elucidated. Thus, the effects of metabolic inducers and inhibitors on nitrosamine metabolism were examined in rat liver, and the relationships between dealkylation and P450 species were analysed.

Suspensions of hepatocytes were incubated with nitrosamines (0.5 mM) for up to 120 min, as reported previously (Kawanishi *et al.*, 1985a). Nitrosamines in incubation mixtures were measured using a gas chromatograph equipped with a nitrogen-phosphate detector. NDMA metabolism was not changed by PB (0.3 mmol/kg on two consecutive days by intraperitoneal injection) or MC (0.075 mmol/kg on two consecutive days by intraperitoneal injection), but was increased by PY (3.0 mmol/kg on three consecutive days by intraperitoneal injection) and EtOH (10% in drinking-water for three days). NBMA metabolism was increased by PB and MC but not by EtOH; PY also increased it slightly. NMBzA metabolism was increased by PB and decreased by PY and EtOH.

Effects of inducers on microsomal dealkylation of nitrosamines are shown in Table 1. Hepatic microsomal dealkylation of nitrosamines was examined by the method of Kawanishi *et al.* (1985b). The results agreed with those found in isolated hepatocytes. PB increased dealkylation of bulky chains, namely NBMA debutylation and NMBzA debenzylation, markedly; NBMA demethylation was also increased. MC increased NBMA debutylation markedly. PY and EtOH selectively increased NDMA demethylation.

The inhibitory effects of SKF 525A (0.1 mM), metyrapone (0.1 mM),  $\alpha$ -naphthoflavone (0.5 mM), PY (1.0 mM) and EtOH (5.0 mM) on microsomal metabolism of nitrosamines were also investigated. It has been reported that SKF and metyrapone selectively inhibit PB-induced drug-metabolizing activities, that  $\alpha$ -naphthoflavone inhibits MC-induced drug-metabolizing activity (Ullrich *et al.*, 1975), and PY inhibits NDMA demethylase (Lake *et al.*, 1982a). In our study, SKF inhibited NDMA demethylation, NBMA demethylation

**Table 1. Effect of pretreatment of inducers on microsomal dealkylation<sup>a</sup>**

Experiment	NDMA demethyl- ation	NBMA		NMBzA		Cytochrome P450 (nmol/mg protein)
		Demethylation	Debutylation	Demethylation	Debenzylation	
(nmol aldehyde/mg protein per min)						
Experiment 1						
Control	0.60±0.06 (5)	0.34±0.04 (4)	0.67±0.06 (4)	1.20±0.08 (4)	2.00±0.25 (4)	0.67±0.02 (10)
PB	0.86±0.07* (6)	0.73±0.08** (4)	2.31±0.31** (4)	0.91±0.11 (4)	6.80±0.68*** (4)	1.38±0.07*** (10)
MC	0.56±0.06 (6)	0.24±0.03 (5)	2.00±0.23** (6)	0.55±0.07** (4)	2.40±0.24 (4)	1.02±0.04** (11)
Experiment 2						
Control	0.75±0.08 (5)	0.49±0.04 (6)	0.84±0.06 (4)	0.99±0.11 (7)	2.04±0.26 (7)	0.84±0.04 (21)
PY	2.15±0.19*** (5)	0.52±0.02 (6)	0.82±0.03 (4)	0.67±0.09* (8)	1.54±0.13 (8)	0.98±0.04** (22)
EtOH	1.33±0.05** (5)	0.54±0.03 (6)	0.77±0.02 (5)	0.91±0.08 (8)	2.14±0.20 (8)	0.86±0.04 (22)

<sup>a</sup>Figures in parentheses, number of animals; \*, significantly different from controls ( $p < 0.05$ ), \*\* ( $p < 0.01$ ), \*\*\* ( $p < 0.001$ )

and debutylation, and NMBzA debenzilation. Metyrapone inhibited NBMA debutylation and NMBzA debenzilation and enhanced NDMA demethylation; it also inhibited NBMA demethylation in PB-treated microsomes.  $\alpha$ -Naphthoflavone inhibited NBMA debutylation and NMBzA debenzilation only in MC-treated microsomes; PY and EtOH inhibited NDMA demethylation most effectively.

These results suggest that dealkylation of nitrosamines is catalysed by several enzymes, which probably depend on P450 species with different specificities to the nitrosamines: PB-induced P450 is active in demethylating NBMA, debutylating NBMA and debenzylating NMBzA; MC-induced P450 is active in debutylating NBMA and debenzylating NMBzA; EtOH-induced P450 and PY-induced P450 are active in demethylating NDMA, although the latter P450 has not been purified and identified. Our preliminary results also show that a purified PB-induced P450 species was active in demethylating and debutylating NBMA and debenzylating NMBzA, and that two purified  $\beta$ -naphthoflavone-induced P450 species were active in debutylating NBMA and debenzylating NMBzA in reconstituted systems (data not shown). It is probable that dealkylating enzymes depending on these P450 species play a role in determining the organ-specific carcinogenicity of nitrosamines. Therefore, carcinogenicity might be changed by inducers of P450, including drugs and alcohol.

# EFFECT OF BUTYLATED HYDROXYANISOLE ON THE METABOLISM OF *N*-NITROSODI-*n*-BUTYLAMINE AND *N*-NITROSOBUTYL(4-HYDROXYBUTYL)AMINE BY RAT HEPATIC S9 PREPARATIONS *IN VITRO*

R. Pastorelli, A. Ancidei, R. Fanelli & L. Airoidi

*Laboratory of Environmental Pharmacology and Toxicology,  
Istituto di Ricerche Farmacologiche 'Mario Negri',  
Via Eritrea 62, 20157 Milan, Italy*

*N*-Nitrosodi-*n*-butylamine (NDBA), the NADPH generating system and various concentrations of butylated hydroxyanisole (BHA) added to rat hepatic S9 fractions resulted in a significant drop (30-50%) in *N*-nitrosobutyl(4-hydroxybutyl)amine (NBHBA) formation and a consequent rise in the amount of substrate recovered unchanged. When NBHBA and NAD<sup>+</sup> were incubated with BHA and S9 fractions, the amount of *N*-nitrosobutyl(3-carboxypropyl)amine (NBCPA) was decreased by 20-40%, and the amount of unmetabolized NBHBA increased.

The food additive BHA reportedly modifies the toxicity and carcinogenicity of chemical carcinogens by interfering with a variety of enzyme systems involved in their metabolism (Kahl, 1984). Only scattered information exists concerning the effect of BHA on the metabolism of nitrosamines (Chung *et al.*, 1984).

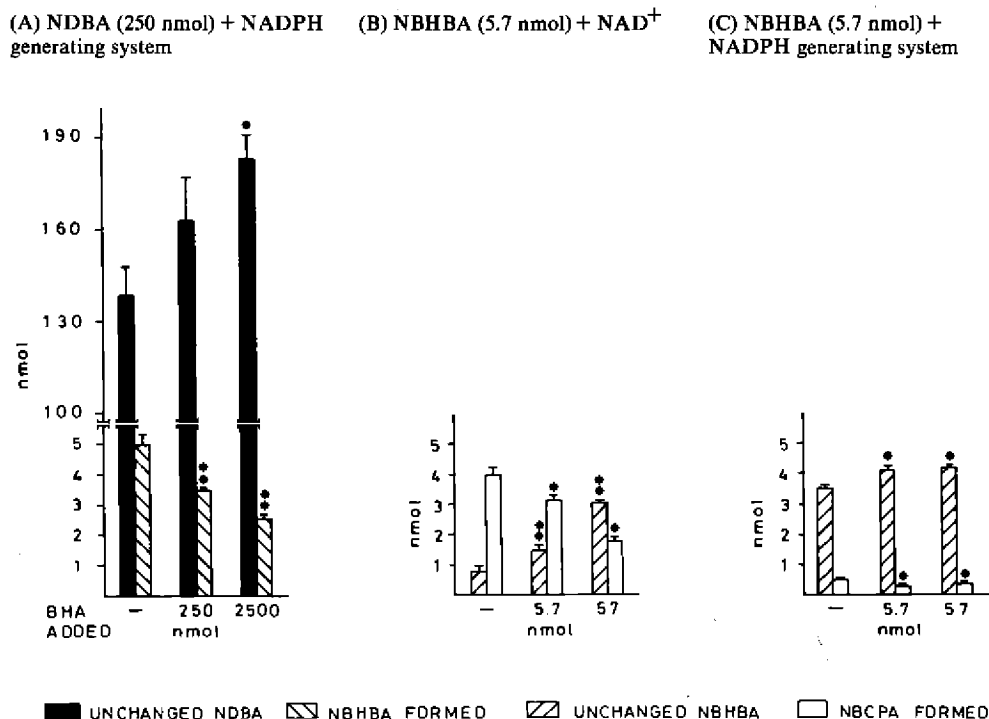
We studied the effect of BHA on the cytochrome P450 (P450)-dependent  $\omega$ -hydroxylation of NDBA to NBHBA, which is reportedly a rat urinary bladder carcinogen (Okada & Ishidate, 1977). The effect of BHA on the further oxidation of NBHBA to NBCPA by the enzymatic system, alcohol/aldehyde dehydrogenase, considered to be responsible for urinary bladder tumour induction in rats (Okada & Ishidate, 1977) was also studied.

## **In-vitro studies**

Medium containing 1 ml of rat hepatic S9 fraction, the NADPH generating system, 0.25 mM NDBA and equimolar or ten-fold molar BHA, was incubated at 37°C for 15 min. To study the effect of BHA on NBHBA metabolism with NAD<sup>+</sup> or the NADPH generating system, a concentration of 5.7  $\mu$ M NBHBA was used, and BHA was added in equimolar or ten-fold molar amounts. Incubations were stopped 5 min after the addition of substrate. NDBA, NBHBA and NBCPA were extracted and analysed by gas chromatography-thermal energy analysis, as described by Airoidi *et al.* (1983b).

The effect on NDBA  $\omega$ -oxidation of BHA added to hepatic S9 mixes is shown in Figure 1A. Addition of equimolar and ten-fold molar BHA to NDBA significantly reduced the amount of NBHBA formed, by 29 and 49% respectively. This finding is in agreement with the reported inhibition of P450-dependent monooxygenase by BHA (Yang *et al.*, 1974). As a result of the decreased  $\omega$ - and other P450-dependent oxidations of NDBA, the amount of NDBA recovered unchanged increased.

**Fig. 1. Effect of BHA on the metabolism of NDBA (A) and of NBHBA (B and C) by rat hepatic S9 fractions.**



Columns represent means  $\pm$  SE of at least four animals; \*,  $p < 0.05$  versus control values; \*\*,  $p < 0.01$  versus control values (Dunnett's test)

Figure 1B shows the effect of addition of BHA *in vitro* on the oxidation of NBHBA to NBCPA in the presence of NAD<sup>+</sup>. BHA reduced the formation of NBCPA by 20-40% and raised the amount of unchanged substrate. This finding confirms the reported inhibition of alcohol dehydrogenase (Wattenberg & Sporn, 1979).

Similar but quantitatively different results were obtained when NBHBA was incubated with the NADPH generating system, added to detect metabolic activities other than alcohol/aldehyde dehydrogenase (Fig. 1C). In the presence of NAD<sup>+</sup> and NADPH, the sum of NBCPA formed and of unchanged NBHBA was the same with or without BHA, indicating that, under our experimental conditions, NBCPA formation is the preferred metabolic pathway for NBHBA.

These results suggest that when BHA is present in the liver simultaneously with the carcinogens NDBA or NBHBA, it might in fact inhibit tumour induction by these compounds. Studies are in progress to assess whether acute or chronic feeding with BHA has similar effects on the metabolism of NDBA and NBHBA.

#### Acknowledgements

This work was supported by the Italian National Council for Research, Special Project 'Oncology' Contract No. 85.02556.44 and by Perfetti S.p.A.

## **REACTION WITH MACROMOLECULES**

# FORMATION AND FATE OF NUCLEIC ACID AND PROTEIN ADDUCTS DERIVED FROM *N*-NITROSO-BILE ACID CONJUGATES

D.E.G. Shuker<sup>1</sup>, J.R. Howell & B.W. Street

*MRC Toxicology Unit, Medical Research Council Laboratories,  
Woodmansterne Road, Carshalton, Surrey SM5 4EF, UK*

<sup>14</sup>C-*N*-Nitrosoglycocholic acid (<sup>14</sup>C-NOGC) reacted with calf thymus DNA *in vitro* to give a number of carboxymethylated adducts (7-carboxymethylguanine [7-CMG], 3-carboxymethyladenine [3-CMA] and *O*<sup>6</sup>-carboxymethylguanine [*O*<sup>6</sup>-CMG]). 7-CMG is excreted unchanged in urine, and its use as a marker for NOGC exposure was studied. Administration of <sup>14</sup>C-NOGC results in a dose-dependent urinary excretion of <sup>14</sup>C-7-CMG and in labelling of blood proteins, albumin and globin. The activity in albumin disappears *in vivo*, with a half-life very similar to that of albumin itself. The monitoring of carboxymethylated nucleic acid bases and proteins appears to be a useful way of monitoring endogenous formation of NOGC and related compounds.

*N*-Nitroso-bile acid conjugates (NBAs; Shuker *et al.*, 1981) are mutagenic and carcinogenic derivatives of the naturally occurring bile acid conjugates (Song Paju *et al.*, 1982; Busby *et al.*, 1985). Intragastric administration of NOGC and *N*-nitrosotaurocholic acid (NOTC) to rats resulted in tumours at several sites, including the liver and glandular stomach.

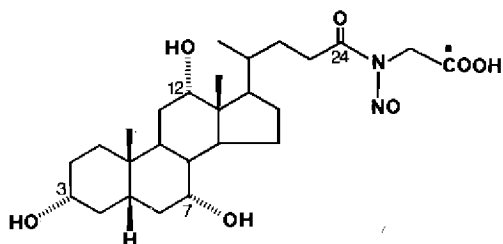
We now report on (1) the characterization of adducts arising from the reaction of NOGC with DNA, (2) the characterization of blood protein binding derived from NOGC, and (3) preliminary results utilizing (1) and (2) to develop methods to monitor exposure to NOGC.

## DNA adducts

<sup>14</sup>C-NOGC (Fig. 1) was synthesized from commercially available <sup>14</sup>C-glycocholic acid (Shuker *et al.*, 1981). <sup>14</sup>C-NOGC (1.6 μmol, 20 μCi) was incubated with calf thymus DNA (400 μg) for 16 h (50 mM phosphate/0.2 mM EDTA; pH, 7.4; 37°C), and DNA was recovered by precipitation with sodium acetate (2.5 M, 0.1 vol) and ethanol (2 vols), centrifugation, followed by two cycles of washing with ethanol and finally dried *in vacuo*. The modified DNA was redissolved in Tris buffer (10 mM; pH, 7.2) and aliquots were taken for hydrolysis under different conditions.

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<sup>1</sup>Present address: International Agency for Research on Cancer, 150 Cours Albert-Thomas, 69372 Lyon Cedex 08, France

**Fig. 1. Structure of  $^{14}\text{C}$ -NOGC**

Neutral thermal hydrolysis ( $70^{\circ}\text{C}$ , 30 min) was followed by precipitation of depurinated DNA and analysis of the supernatant by reverse-phase high-performance liquid chromatography (HPLC) (see Fig. 2 for details). By comparison with authentic standards, the two radioactive peaks in the chromatogram corresponded to 7-CMG and 3-CMA. The amounts of each adduct formed were (as a percentage of  $^{14}\text{C}$ -NOGC) 0.025% and 0.006%, respectively, or 980 pmol 7-CMG/mg DNA and 234 pmol 3-CMA/mg DNA.

Acid hydrolysis of the modified DNA (0.1 M HCl,  $70^{\circ}\text{C}$ , 30 min) resulted in the appearance of two additional peaks in the HPLC chromatogram (Fig. 2). The later eluting peak corresponded to *O*<sup>6</sup>-CMG (0.0026%, 104 pmol/mg DNA). The earlier peak ('A') has not yet been identified, but appears to be unstable in strong acid (1 M perchloric acid,  $70^{\circ}\text{C}$ , 30 min).

Interestingly, *O*<sup>6</sup>-CMG has recently been found to be completely resistant to repair by bacterial and mammalian *O*<sup>6</sup>-alkylguanine repair proteins (Shuker, Brennan and Margison, unpublished data). If *O*<sup>6</sup>-CMG is a mutagenic lesion, similar to *O*<sup>6</sup>-methylguanine, then its lack of repair is particularly interesting in consideration of the mechanism of carcinogenesis of NOGC and related compounds.

### Blood protein binding

Different doses of  $^{14}\text{C}$ -NOGC (4.73, 9.46 and 18.92  $\mu\text{Ci}$ ) were administered intragastrically to rats (female LAC:P, 190-210 g). After eight days, dose-dependent levels of radioactivity were associated with both plasma and red blood cells (Fig. 3). In plasma, most of the activity (> 90%) was associated with albumin (as determined by affinity chromatography on Active Blue Sepharose). Similarly, binding to globin accounted for most of the radioactivity associated with red blood cells.

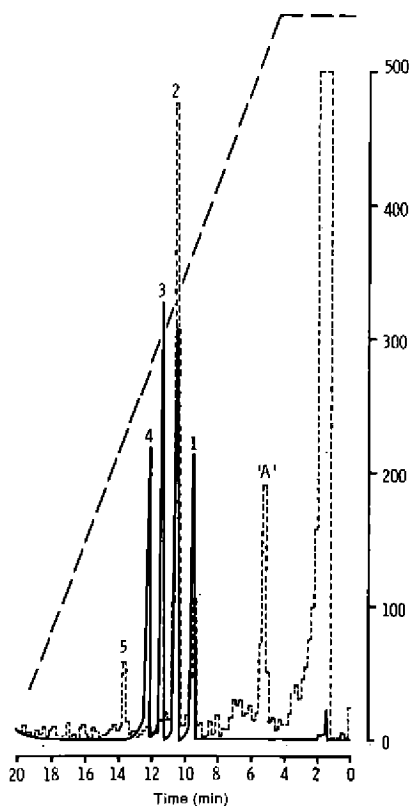
In order to examine the clearance of albumin-bound radioactivity, two rats were dosed with  $^{14}\text{C}$ -NOGC (10  $\mu\text{Ci}$ , intragastrically), and blood samples were taken at regular intervals. Plasma radioactivity was measured over a 13-day period and found to disappear with a half-life of 2.56 days, which is very close to the value for the half-life of albumin in rats (2.66 days; Schreiber *et al.*, 1971).

When the radioactive albumin was hydrolysed and the amino acids separated by HPLC, it was found that glycine and serine contained the radioactivity. Thus, metabolic incorporation rather than covalent binding was responsible for the activity (Gan, Shuker & Tannenbaum, unpublished data).

### Detection of carboxymethylation *in vivo*

The major DNA adduct formed from NOGC is 7-CMG, and its detection *in vivo* could be the basis for a dosimetric method. By analogy with other 7-alkylguanines, 7-CMG would be expected to be excreted in urine.

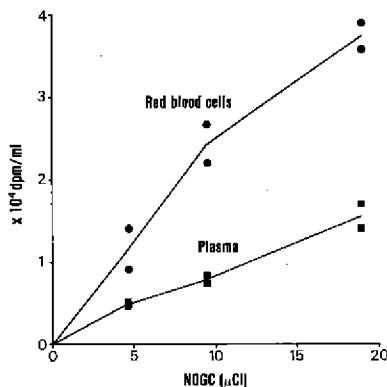
**Fig. 2.** Reverse-phase HPLC chromatogram of 0.1 N HCl hydrolysate from  $^{14}\text{C}$ -NOGC-treated calf thymus DNA



—, absorbance at 278 nm; —,  $^{14}\text{C}$  dpm. Column: Ultrasphere - ODS ( $5\ \mu\text{m}$ )  $150 \times 4.6$  mm ID. Solvent system; 0.1% v/v aqueous heptafluorobutyric acid/methanol. Flow rate, 1 ml/min. Peaks: 1, 3-CMA; 2, 7-CMG; 3, guanine; 4, adenine; 5,  $O^6$ -CMG; 'A', unknown. Authentic standards of 3-CMA and 7-CMG were added to DNA prior to hydrolysis.

Preliminary results indicate that dose-dependent urinary excretion of 7-[2- $^{14}\text{C}$ ]-CMG occurs following administration of  $^{14}\text{C}$ -NOGC, the alkylated guanine representing about 0.046% of the dose on a molar basis. In comparison, methylating agents derived from *N*-nitroso compounds afforded urinary 7-methylguanine levels reflecting 0.012 to 0.014% of the administered dose (Farmer *et al.*, 1986).

**Fig. 3.**  $^{14}\text{C}$  Activity associated with blood components eight days after dosing with various levels of  $^{14}\text{C}$ -NOGC



Blood was collected by cardiac puncture under ether anaesthesia into heparinized tubes and centrifuged. Plasma was collected and the blood cells were washed (three times) with saline. Aliquots ( $100\ \mu\text{l}$ ) of plasma and packed red blood cells were assayed for  $^{14}\text{C}$  activity by combustion to  $^{14}\text{C}$ - $\text{CO}_2$  followed by scintillation counting. Two animals were used at each dose level.

7-[2- $^{14}\text{C}$ ]-CMG (for synthesis, see Howell, 1985) was found to be excreted in rats, *via* the urine, essentially unchanged ( $> 98\%$  as determined by HPLC). The level of excretion was dependent on the route of administration. Intra-gastric instillation afforded mainly faecal excretion (82%) with the balance in the urine, whereas intraperitoneal dosing resulted primarily in urinary clearance (82%). In either case, no expired  $^{14}\text{C}$ - $\text{CO}_2$  was detected. Since the intraperitoneal route probably more accurately reflects the fate of 7-CMG arising from DNA alkylation, its urinary excretion appeared to be a promising indicator of carboxymethylation *in vivo*.



**Conclusions**

The determination of carboxymethylated nucleic acid bases appears to be a promising approach for the detection of NOGC *in vivo*. The natural occurrence of carboxymethylated adducts is not very likely (Farmer *et al.*, 1986); therefore, the sensitivity of such a monitoring method would be limited only by the analytical methodology. However, carboxymethyl adducts could arise from other sources (e.g., C-terminal *N*-nitroso glycyl peptides [Challis *et al.*, 1984] or azaserine-like compounds [Zurlo *et al.*, 1982]), so that unambiguous detection of NBAs *in vivo* would require the concomitant determination of 2-sulfoethyl adducts derived from the taurine moiety of NOTC.

Preliminary experiments utilizing albumin as an alkylation dosimeter would appear to indicate that it is unsuitable for monitoring exposure to NOGC.

**Acknowledgements**

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## A METHOD TO DETERMINE THE CARBAMOYLATING POTENTIAL OF 1-(2-CHLOROETHYL)-1-NITROSOUREAS

W. Stahl<sup>1</sup>, R.L. Krauth-Siegel<sup>2</sup>, R.H. Schirmer<sup>3</sup> & G. Eisenbrand<sup>1,3</sup>

<sup>1</sup>*Department of Food Chemistry and Environmental Toxicology, University of Kaiserslautern, P.O. Box 3049, D-6790 Kaiserslautern; and*

<sup>2</sup>*Institute of Biochemistry II, University of Heidelberg, Im Neuenheimer Feld 328, D-6900 Heidelberg, Federal Republic of Germany*

1-(2-Chloroethyl)-1-nitrosoureas (CNU) are alkylating agents that also possess carbamoylating activity, depending on the chemical nature of the substituent at N-3. Although effects on a variety of enzymes, including inhibition of glutathione reductase (GS-R) have been attributed to carbamoylation, the biological significance is still not well understood. This deficiency is due at least in part to the analytical method that has been used to measure carbamoylation: in-vitro reaction with the  $\omega$ -amino group of lysine. Reaction of CNU with glutathione (GSH) offers a better estimation of carbamoylating potential *in vitro*. The decrease in free thiol groups during incubation of GSH with various CNU can be followed using the thiol reagent 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). With this test, carbamoylating potential relative to that of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) as 100% was 94% for 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea (MeCCNU), 86% for 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU), 16% for 1-(2-chloroethyl)-3-(2-hydroxyethyl)-1-nitrosourea (HECNU) and 6% for chlorozotocin (CLZ). Various carbamoylated and alkylated GSH derivatives, such as *S*-(2-chloroethylcarbamoyl)-, *S*-(2-hydroxyethylcarbamoyl)-, *S*-(cyclohexylcarbamoyl)-, *S*-(4-methylcyclohexylcarbamoyl)- and *S*-(2-hydroxyethyl)glutathione, are formed on incubation of GSH with CNU. High-performance liquid chromatography (HPLC) revealed that, in comparison to carbamoylated compounds, alkylated GSH derivatives are formed in only low yields (< 3%). Formation of carbamoylated products during incubation correlated with the decrease in free thiol groups. Concentrations achieving 50% GS-R inhibition *in vitro* were 0.16 mM for BCNU and 1.9 mM for HECNU. Work in progress suggests that in the case of BCNU *S*-carbamoylation of human erythrocyte GS-R at Cys<sub>58</sub> is responsible for inhibition, whereas in the case of HECNU exclusively 2-hydroxyethylation of Cys<sub>58</sub>-SH is detectable.

CNU are alkylating agents that also exhibit carbamoylating activity, depending on the chemical nature of the substituent at N-3. Inhibiting effects on a variety of enzymes have been attributed to carbamoylation. The most prominent effect appears to be a severe and generalized GS-R deficiency, which has been described after treatment with BCNU (Frischer & Ahmad, 1977). The biological significance of carbamoylation is, however, not well understood. The analytical procedures that have been used to assess carbamoylating activity mostly rely on an in-vitro reaction with the  $\omega$ -amino group of lysine or polylysine (Wheeler *et al.*, 1975). The carbamoylation of amino groups might, however, have only

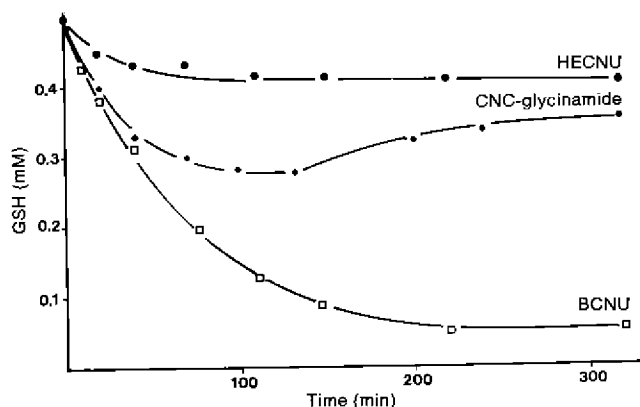
<sup>3</sup> To whom correspondence should be addressed

limited predictive value for in-vivo effects. Reaction of CNUs with biologically relevant sulfhydryl (SH) groups appears to be more important. We have therefore developed a new carbamoylation test based on the reaction of nitrosoureas with the predominant non-protein thiol GSH.

### Incubation of GSH with CNUs

Compounds tested were BCNU, MeCCNU, CCNU, *N*'-(2-chloroethyl)-*N*'-nitrosoglycinamide (CNC-glycinamide), HECNU and CLZ. Compounds were incubated at pH 7.2 with an equimolar amount of GSH (0.5 mM/0.5 mM), and the concentration of free thiol groups was determined at different times. As an example, Figure 1 shows the time-dependent disappearance of GSH on reaction with BCNU, HECNU and CNC-glycinamide. At  $t_{1/2}$  (time at which 50% of the respective CNU had decomposed), the extent of thiol consumption (mol %) was: BCNU, 49%; MeCCNU, 46%; CCNU, 42%; CNC-glycinamide, 25%; HECNU, 8%; and CLZ, 3%. In the case of CNC-glycinamide, a re-liberation of free SH groups, up to almost the original amount, was observed. The unexpected reappearance of free GSH after reaction with CNC-glycinamide can be attributed to a ring-closure reaction that results in the elimination of hydantoin (Stahl & Eisenbrand, unpublished data).

**Fig. 1. Time-dependent disappearance of GSH after reaction with BCNU, HECNU and CNC-glycinamide**



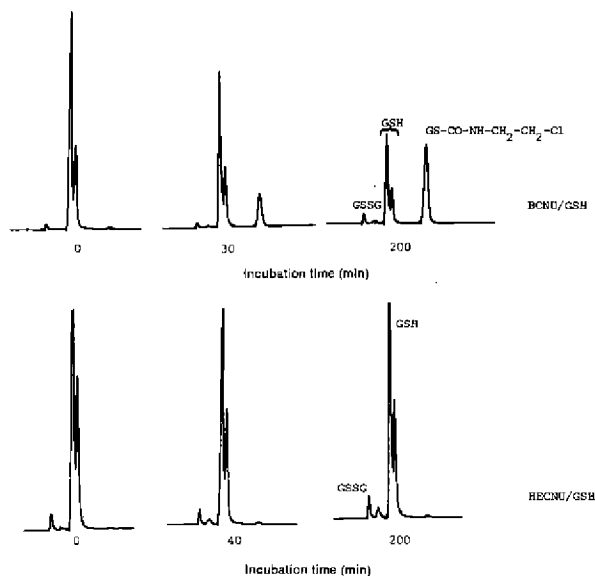
5  $\mu$ M CNU (dissolved in 200  $\mu$ l tetrahydrofuran) incubated with 5  $\mu$ M GSH (dissolved in 9.8 ml sodium/potassium phosphate buffer pH 7.2 containing 100  $\mu$ l 0.1 M EDTA). GSH was determined with 5,5'-dithiobis(2-nitrobenzoic acid) (Ellmann, 1959).

### Identification of GSH conjugates

Reaction products in the incubation mixtures were analysed by HPLC (Fig. 2). We identified *S*-(2-chloroethyl)-carbamoyl-, *S*-(cyclohexyl)-carbamoyl-, *S*-(4-methylcyclohexyl)-carbamoyl- and *S*-2-(hydroxyethyl)-carbamoylglutathione as the main adducts from the reaction of GSH with BCNU, CCNU, MeCCNU and HECNU, respectively. GSH conjugates resulting from alkylation were found in only minor yields (< 3%). Thus, the observed loss of free thiol groups resulted practically exclusively from carbamoylation of GSH. Oxidation to oxidized GSH occurred to only a very minor extent under these conditions.

The results show that there are distinct differences in the reactivity of CNUs towards GSH-SH groups. HECNU and CLZ are practically not carbamoylating under these conditions, whereas BCNU and CCNU are strong carbamoylators. The differences between

**Fig. 2. HPLC comparison of incubation mixtures of GSH with BCNU and HECNU at different times**



Column, Shandon ODS Hypersil  $5\mu\text{m}$ ; solvent, acetonitrile/citrate buffer (pH 3, 0.1 M); derivatization, Fluorescamin (Floram); detection, fluorescence Ex 390 nm, Em 475 nm; GSSG, oxidized GSH

BCNU and HECNU confirm results from studies on *in-vitro* inhibition of GS-R, which showed that *in vitro* BCNU is a much stronger GS-R inhibitor than HECNU (Schirmer *et al.*, 1984). In the case of BCNU, *S*-carbamoylation of human erythrocyte GS-R at Cys<sub>58</sub> appears to be responsible for inhibition. In the case of HECNU, exclusively *S*-alkylation (2-hydroxyethylation) of Cys<sub>58</sub>-SH was detected (Krauth-Siegel *et al.*, 1986).

# ALKYLATION OF 2'-DEOXYTHYMIDYL(3' → 5')-2'-DEOXYTHYMIDINE WITH 2-HYDROXYETHYLNITROSOUREA AND STABILITY OF THE RESULTING PHOSPHOTRIESTER

N. Müller, J. Conrad & G. Eisenbrand<sup>1</sup>

*Department of Food Chemistry and Environmental Toxicology,  
University of Kaiserslautern, Federal Republic of Germany*

One of the main targets of RNA and DNA alkylation is the phosphate group. In contrast to RNA phosphotriesters, DNA phosphotriesters are relatively stable. Introduction of 2-hydroxyethyl phosphotriesters into DNA, however, has been reported to decrease its stability towards hydrolytic cleavage considerably. 2-Chloroethylnitrosooureas (CNU) have been found to form predominantly 2-hydroxyethyl adducts of DNA. In the present study, we have determined the stability of 2'-deoxythymidylyl-(3' → 5')-2'-deoxythymidine (dTpdT) after alkylation with various nitrosooureas, including *N*-methyl-*N*-nitrosoourea (MNU) and 2-hydroxyethylnitrosoourea (HENU) at alkaline and neutral pH (at 37°C). The half-lives ( $t_{1/2}$ ) at pH 12.5 were, for example, 2.8 h for di(2'-deoxythymidine)methyl-phosphotriester [dTp(me)dT] but < 1 min for di(2'-deoxythymidine)(2-hydroxyethyl)-phosphotriester [dTp(he)dT]. At pH 7.0, dTp(me)dT was stable for more than three days, whereas the  $t_{1/2}$  for dTp(he)dT was only 1.0 h at pH 7.0 (27 min at pH 9.0). The marked lability of dTp(he)dT, in comparison to other phosphotriester analogues, can be explained by intermediate formation of a dioxaphospholane ring resulting in triester bond breakage. Our data strongly support the hypothesis that a great proportion of DNA single-strand breaks induced in DNA by HENU or CNU can be attributed to intermediate formation of 2-hydroxyethyl phosphotriesters.

One of the main targets of RNA and DNA alkylation is the phosphate group (Singer, 1976). In contrast to RNA phosphotriesters, DNA phosphotriesters are relatively stable. Introduction of 2-hydroxyethyl phosphotriesters into DNA, however, has been reported to decrease its stability towards hydrolytic cleavage considerably (Wallis & Ehrenberg, 1968). *In vitro*, CNU have been found to form predominantly 2-hydroxyethyl adducts of DNA nucleobases (Tong *et al.*, 1982).

In the present study, we have determined the stability of dTpdT after alkylation with MNU and HENU at alkaline and neutral pH (at 37°C). The  $t_{1/2}$  at pH 12.5 were, for example, 2.8 h for dTp(me)dT but ≤ 1 min for dTp(he)dT. We conclude that most of the DNA single-strand breaks induced in DNA by HENU or CNU can be attributed to intermediate formation of 2-hydroxyethyl phosphotriesters.

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<sup>1</sup>To whom correspondence should be addressed

## Results and discussion

Phosphate alkylation of dTpdT with MNU or HENU brings about a diastereomeric pair of dTp(alk)dT (alk = methyl, 2-hydroxyethyl), because the covalent attachment of the alkyl moiety to the phosphate group of the dideoxythymidine monophosphate can occur at either of the two available oxygen atoms. Formation of diastereomeric phosphotriesters was reported previously by Jensen and Reed (1978) after alkylation of poly(dA-dT) with *N*-ethyl-*N*-nitrosourea.

The reaction of dTpdT with MNU (molar ratio, 1:550) yields 5% of dTp(me)dT isomers in the reaction solution (90% unreacted dinucleotide). Alkylation of dTpdT with HENU (1:550) results in 2% of dTp(he)dT isomers and 96% dTpdT. Further high-performance liquid chromatographic peaks (< 1% of total adducts) most probably result from *N*- or *O*-alkylated products of dTpdT, but could not be identified.

The stabilities of these phosphotriesters differ drastically, as shown in Table 1. The strongly increased instability of the 2-hydroxyethylated triester can be ascribed to alkali-catalysed formation of a dioxaphospholane intermediate, which decomposes to phosphodiester, (2'-deoxythymidine)-5'-(2-hydroxyethyl)phosphate, or (2'-deoxythymidine)-3'-(2-hydroxyethyl)phosphate and 2'-deoxythymidine (Fig. 1).

**Table 1. Half-lives ( $t_{1/2}$ ) and second-order rate constants ( $K_2$ ) for the decomposition of alkylated dTpdT at different pH values and 37°C**

Compound	pH	$t_{1/2}^a$ (min)	$K_2^a$ (l/M sec)
dTp(me)dT	12.5	165 <sup>b</sup>	$2.22 \times 10^{-3}$
	7.0	stable <sup>c</sup>	
dTp(he)dT	12.5	$\leq 1$	$\leq 3.7_d \times 10^{-1}$
	9.0	27	-
	7.0	60	-

<sup>a</sup>The  $t_{1/2}$  of the decomposition reactions can be determined in a  $\ln c/t$  diagram; the following equation was applied to determine the bimolecular rate constants  $K_2$ :

$$K_2 = \ln 2 / (3.16 \times 10^{-2} t)$$

<sup>b</sup>See Swenson *et al.* (1976);  $t_{1/2} = 158 \pm 6$  min (37°C, pH 13)

<sup>c</sup>Stable for more than 3 days

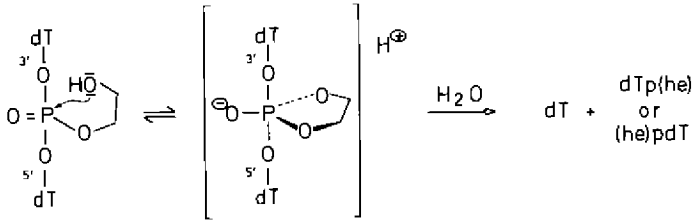
<sup>d</sup>Cannot be calculated because the concentrations of alkali and of the phosphotriesters are of the same order.

2-position of a phosphotriester will labilize the sugar phosphate backbone similarly. Accordingly, 2-hydroxypropyl or longer alkyl groups as well as those resulting from alkylation by substituted oxiranes should bring about DNA strand breaks rapidly.

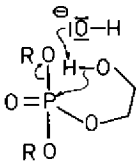
The model compound diethyl 2-methoxyethyl phosphate has been found to be very stable in alkaline conditions, whereas its 2-hydroxy congener is highly unstable (Lown & McLaughlin, 1979; Conrad, 1985). This confirms that a free hydroxy group in the 2-position is responsible for the rapid decomposition of 2-hydroxyethyl phosphates in DNA.

As a result of 2-hydroxyethylation of phosphodiester in DNA and subsequent dioxaphospholane ring closure, cleavage of the phosphate-sugar bond should result in single-strand breaks in DNA (Fig. 2). Such breaks should appear much more rapidly than those observed after alkylation of nucleobases and/or generated during repair. These differences in the kinetics of strand-break formation have been described by Lown and McLaughlin (1979) as type I and type II single-strand scission. It is conceivable that any hydroxy group introduced into the

**Fig. 1. Decomposition of the di(2'-deoxythymidine)-2-hydroxyethyl phosphotriester; dTp(he), (2'-deoxythymidine)-3'-(2hydroxyethyl)phosphate; (he)pdT, (2'-deoxythymidine)-5'-(2hydroxyethyl)phosphate**



**Fig. 2. Base-catalysed formation of a dioxaphospholane ring**



Hydroxyethylating nitrosoureas have been shown to have no or poor antileukaemic activity in rodent leukaemias, in contrast to their chloroethylating and cross-linking counterparts, which are highly effective (Zeller *et al.*, 1985). 1-(2-Hydroxyethyl)-1-nitrosoureas have been found to be strong mutagens and carcinogens (Lijinsky & Reuber, 1983; Lijinsky *et al.*, 1985). Hydroxyethylation therefore appears to be a lesion more relevant for malignant transformation than for antitumour efficiency.

#### Acknowledgements

This investigation was supported by the German Ministry for Research and Technology (BMFT), project number PTB 03 8709 1. MNU was a gift from Mr Otto Zelesny, German Cancer Research Center, Heidelberg, FRG.

# MECHANISM OF INHIBITION OF N-METHYL-N-NITROSOUREA-INDUCED MUTAGENICITY AND DNA BINDING BY ELLAGIC ACID

R. Dixit & B. Gold

*Eppley Institute for Research in Cancer and Allied Diseases,  
University of Nebraska Medical Center,  
Omaha, Nebraska 68105, USA*

Ellagic acid (EA) is a dilactone derivative of shikimic acid, which is found in a variety of soft fruits and vegetables. EA inhibits mutagenesis and carcinogenesis induced by benzo[*a*]pyrene and its bay-region dihydrodiol epoxide derivative by preventing their covalent binding to DNA. EA at concentrations of 100, 250, 500 and 1000 nmol/plate inhibited the mutagenicity of *N*-methyl-*N*-nitrosourea (MNU) (400 nmol/plate) in *Salmonella typhimurium* TA100 by 3, 13, 45 and 60%, respectively. A study of inhibition of <sup>3</sup>H-MNU-mediated DNA methylation by EA showed that it inhibited only the formation of *O*<sup>6</sup>-methylguanine, while attack at the N7 and N3 positions of guanine and adenine, respectively, was not altered. This inhibition was observed only in double-stranded DNA. Ultraviolet and equilibrium dialysis studies show that EA has a definite affinity for DNA, but that an intercalating process is not involved.

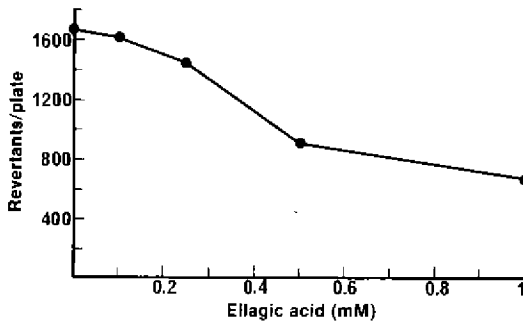
EA, 2,3,7,8-tetrahydroxy[1]benzopyrano[5,4,3-*cde*][1]benzopyran-5,10-dione, a natural shikimate derivative present in soft fruits and vegetables (Bate-Smith, 1972), inhibits the carcinogenicity, mutagenicity and DNA binding (Wood *et al.*, 1982; Lesca, 1984; Chang *et al.*, 1985) of benzo[*a*]pyrene-7,8-dihydrodiol 9,10-epoxide by accelerating its hydrolysis (detoxification) *via* general base catalysis (Sayer *et al.*, 1982). Prevention of MNU- and *N*-nitrosodimethylamine (NDMA)-induced mutagenicity and DNA binding is discussed in terms of a proposed EA-duplex DNA-affinity binding mechanism.

## Mutagenicity studies

MNU-induced mutations to histidine-independent growth were assessed in *S. typhimurium* strain TA100 (Maron & Ames, 1983). Bacterial suspensions in 0.5 ml of 5 mM potassium phosphate buffer pH 7.4 were preincubated for 10 min with dimethyl sulfoxide (DMSO; 20  $\mu$ l) or with EA in DMSO (20  $\mu$ l), at final concentrations of 0.05, 0.10, 0.25, 0.50 and 1.00 mM. At the end of preincubation, 400 nmol MNU (final concentration, 0.40 mM) were added to the incubation mixture. After 48 h incubation, revertants were scored; all data are derived from two separate experiments performed in triplicate. The studies with NDMA were carried out using essentially the same procedure, except that pyrazole-induced 9000  $\times$  *g* microsomal supernatant (S9) from eight- to ten-week-old male Sprague-Dawley rats (Tu *et al.*, 1981) was included for activation. Final concentrations of 25, 50, 100 and 200 mM NDMA were used in the presence of 8.7 mg S9 protein. The results of these studies, which indicate an inhibitory activity of EA, are shown in Figures 1 and 2.

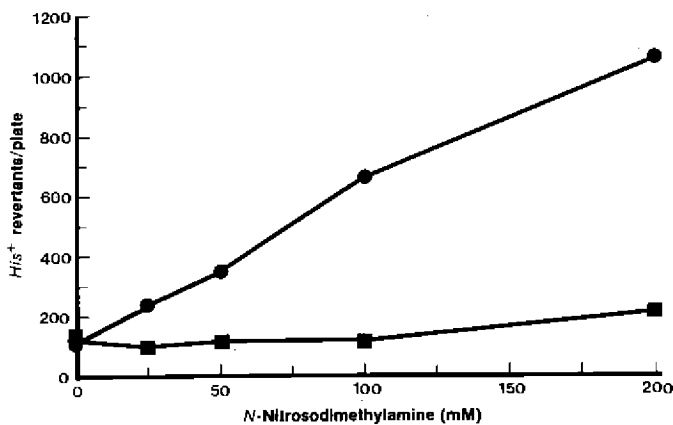


**Fig. 1. Effect of EA on mutagenicity of MNU in *S. typhimurium* TA100**



Mutagenicity of 0.40 mM MNU in the absence of EA was  $1666 \pm 146$  histidine-independent revertants per plate; mutagenicity of 20  $\mu$ l DMSO (control) was  $135 \pm 25$  revertants per plate. Values are the mean of two triplicate determinations

**Fig. 2. Effect of EA (3 mM) on the mutagenicity of NDMA in *S. typhimurium* TA100 in the presence of pyrazole-induced (8.7 mg protein) rat liver S9**



Mutations/plate without (●) and with (■) EA are presented. Background mutation rate with 20  $\mu$ l DMSO was  $124 \pm 15$  revertants/plate. Data points represent the mean of two triplicate determinations.

### Methylation of DNA by <sup>3</sup>H-MNU

Salmon sperm DNA (double-stranded), single-stranded DNA and polydeoxynucleotide (double-stranded) were incubated in 50 mM Tris buffer (pH 7.4) with <sup>3</sup>H-MNU (10  $\mu$ Ci, 0.66 mM) and DMSO or EA in DMSO (0.72, 1.32, 2.64 and 6.60 mM) in 0.3 ml total volume at 37°C overnight, resulting in complete hydrolysis of MNU. The pH of the incubation mixture remained constant during the entire incubation period, at which time the DNA or polydeoxynucleotide was precipitated overnight and washed repeatedly with cold 95% ethanol and acetone. MNU-treated double- and single-stranded DNA and polydeoxynucleotide were hydrolysed to purine bases in 0.5 ml of 0.1 N HCl for 20 h at 37°C. The acid hydrolysate, containing free purine bases, was neutralized by adding ammonium hydroxide. Alkylated purine bases were fractionated by high-performance liquid chromatography,

essentially as described by Morimoto *et al.* (1983). The results of these studies are shown in Tables 1 and 2. EA specifically blocks alkylation at the O<sup>6</sup> position of guanine, with little or no blockage at N7 of guanine or N3 of adenine. Significantly, EA inhibits alkylation only of double-stranded DNA. Data on the DNA affinity binding of EA are shown in Table 3.

**Table 1. Effect of EA on the methylation of DNA by <sup>3</sup>H-MNU**

EA (mM)	DNA adducts (pmol/μmol nucleotide) <sup>a</sup>		
	N7-methyl	O <sup>6</sup> -methyl	O <sup>6</sup> :N7 ratio
0	103.3 ± 4.0 <sup>b</sup>	20.0 ± 4.3	0.19
0.72	111.5 ± 4.3	20.1 ± 4.0	0.18
1.32	82.6 ± 3.2	12.7 ± 2.7	0.15
2.64	83.6 ± 3.2	7.1 ± 1.5	0.08
6.60	84.6 ± 2.6	3.3 ± 0.3	0.04

<sup>a</sup>Incubation mixture in a total of 300 μl contained 50 mM Tris buffer (pH 7.4), MNU (0.66 mM), 10 μCi <sup>3</sup>H-MNU, salmon sperm DNA (0.9 mM nucleotide) and DMSO or EA in DMSO. Reaction mixture was incubated overnight with gentle shaking at 37°C.

<sup>b</sup>Values are the means of three determinations ± standard deviation.

**Table 2. Effect of EA on MNU-mediated<sup>a</sup> methylation of double-stranded (ds) and single-stranded (ss) DNA and poly(dG-dC)**

DNA	EA <sup>b</sup>	DNA adducts (pmol/μmol nucleotide)		
		N7-guanine	O <sup>6</sup> -guanine	O <sup>6</sup> :N7 ratio
ds	—	231.3 ± 26.6 <sup>c</sup>	25.6 ± 2.0	0.11
ds	+	178.6 ± 20.0	10.6 ± 0.7	0.05
ss	—	76.6 ± 26.0	9.3 ± 1.6	0.12
ss	+	84.0 ± 10.6	12.0 ± 2.0	0.14
dG-dC <sup>d</sup>	—	120.3 ± 10.0	8.7 ± 0.3	0.07
dG-dC	+	80.0 ± 6.0	3.7 ± 0.2	0.04

<sup>a</sup><sup>3</sup>H-MNU concentration, 0.66 mM (10 μCi)

<sup>b</sup>EA concentration, 1.3 mM, when used (+)

<sup>c</sup>Values are the means of three determinations ± standard deviation.

<sup>d</sup>Length of oligomer, 12-18 nucleotides

**Table 3. Noncompetitive binding of <sup>3</sup>H-EA to nucleic acids<sup>a</sup>**

Nucleic acid	EA binding (pmol/μmol nucleotide)
Double-stranded DNA (3 mM)	1233 ± 57 <sup>b</sup>
Single-stranded DNA (3 mM)	1133 ± 33
poly(dG-dC) (15 mM)	1866 ± 260
poly(dA-dT) (15 mM)	2733 ± 533
RNA (3 mM)	1533 ± 57

<sup>a</sup>EA concentration, 7 μM

<sup>b</sup>Values are the means of three determinations ± standard deviation.

### Acknowledgement

This work was supported by a grant from the University of Nebraska Medical Center and NIH Laboratory Cancer Research Center Grant P30 CA36727.

# COMPARISON OF INDUCTION OF DNA SINGLE-STRAND BREAKS AND INITIATION OF RAT HEPATOCARCINOGENESIS BY DIFFERENT NITROSAMINES

V. Pr at<sup>1,3</sup>, C. Schulze<sup>1</sup>, W. Sterzel<sup>2</sup>, G. Eisenbrand<sup>2</sup> & M. Roberfroid<sup>1</sup>

<sup>1</sup>*Unit  de Biochimie Toxicologique et Canc rologique, Universit  Catholique de Louvain, UCL 7369, 1200 Brussels, Belgium; and Department of Food Chemistry and Environmental Toxicology, University of Kaiserslautern, 6750 Kaiserslautern, Federal Republic of Germany*

The capacities of nitrosamines to induce DNA single-strand breaks (SSB) and to initiate carcinogenesis in rat liver were compared. *N*-Nitrosodiethanolamine (NDELA), *N*-nitrosoethylhydroxyethylamine (NEHEA) and *N*-nitrosodiethylamine (NDEA) were equipotent in inducing DNA SSB when administered by gavage at doses of 0.35 mmol/kg, 0.015 mmol/kg and 0.37 mmol/kg, respectively. Male Wistar rats were injected with these nitrosamines and were then submitted to a selection procedure. Ten rats per group were sacrificed one week after the end of the selection to see the effects of the nitrosamines on the development of preneoplastic lesions. The numbers of  $\gamma$ -glutamyl transferase (GGT)-positive lesions per cm<sup>2</sup> were 0.8, 2.1, 5.2 and 40.1 in rats treated with saline, NDELA, NEHEA and NDEA, respectively. *N*-Nitrosobis(2,2,2-trifluoroethyl)amine (6F-NDEA), a nongenotoxic and noncarcinogenic nitrosamine, induced 0.7 GGT-positive lesions per cm<sup>2</sup>. Ten rats per group also received 0.05% phenobarbital in their drinking-water and were killed six months after initiation in order to see the effect of the different nitrosamines on the incidence and yield of tumours. Two extrahepatic cancers were found after administration of NEHEA, whereas two hepatocellular carcinomas were detected after injection of NDEA. No cancer developed in the other groups. Although other factors may influence the process, these results indicate that no simple correlation can be established between induction of SSB in DNA and initiation of tumours by nitrosamines in rat liver.

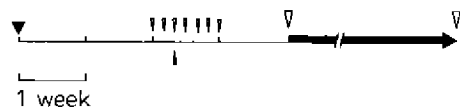
It is generally assumed that complete carcinogens are genotoxic and that carcinogenesis is initiated by alterations to DNA. The aim of the present work was to determine whether there is a correlation between the induction of DNA SSB and the initiating effect of nitrosamines in rat liver.

DNA SSB induced in rat liver by the nitrosamines were measured using the alkaline elution technique (Sterzel *et al.*, 1985). Equipotent oral doses of the nitrosamines tested were estimated to be 0.35 mmol/kg NDELA, 0.015 mmol/kg NEHEA and 0.37 mmol/kg NDEA (Sterzel, 1986). 6F-NDEA, a nongenotoxic and noncarcinogenic nitrosamine (Preussmann *et al.*, 1981), was tested for comparison. The initiating activity of the nitrosamines with regard to rat hepatocarcinogenesis was determined by quantification of foci and nodules in early stages and of liver cancers in later stages (Pr at *et al.*, 1986).

<sup>3</sup>Charg  de Recherches F.N.R.S. (Belgium)

The experimental protocol used is schematized in Figure 1 (Lans *et al.*, 1983). Briefly, it consists of treating male Wistar rats by gavage with a single dose of saline, NDELA (0.35 mmol/kg), NEHEA (0.015 mmol/kg), NDEA (0.37 mmol/kg) or 6F-NDEA (0.37 mmol/kg), given as an aqueous solution. Two weeks later, the rats were submitted to a modified selection procedure (Solt & Farber, 1976), consisting of seven daily gavages of 6 mg 2-acetylaminofluorene and 2 ml/kg carbon tetrachloride given on day 3. One week after the end of the selection, ten rats per group were killed to analyse the effect of the nitrosamines on the development of foci and nodules (Solt & Farber, 1976). The remaining rats received 0.05% phenobarbital in their drinking-water and were sacrificed after 25 weeks to see the effect of the nitrosamines on cancer development (Pr at *et al.*, 1986). This triphasic protocol was shown previously to accelerate the appearance of liver cancers.

**Fig. 1. Experimental protocol used to analyse the effects of different nitrosamines on the development of foci, nodules and cancers in rat liver**



▼, saline, NDELA, NEHEA, NDEA or 6F-NDEA intragastrically; ▽, 2-acetylaminofluorene, 6 mg intragastrically; ▲, carbon tetrachloride, 2ml/kg intragastrically; ■, phenobarbital, 0.05% in drinking-water; ▽, sacrifice

(data not shown). With regard to tumour induction after 25 weeks, one of ten NEHEA-treated rats had a kidney cancer, which metastasized, and another had a salivary gland adenocarcinoma; two of ten NDEA-treated rats had a hepatocellular carcinoma. No tumour was found in the other groups.

**Table 1. Quantitative analysis of the effects of NDELA, NEHEA, NDEA and 6F-NDEA on the development of GGT-positive lesions after five weeks**

Treatment	No. of animals	GGT-positive lesions (%)	No. of GGT-positive lesions/cm <sup>2</sup>
Saline	9	0.04 ± 0.03	0.8 ± 0.4
NDELA	10	0.26 ± 0.07 <sup>a</sup>	3.1 ± 0.7 <sup>a</sup>
NEHEA	10	0.22 ± 0.08 <sup>a</sup>	5.2 ± 1.7 <sup>a</sup>
NDEA	11	2.67 ± 0.62 <sup>a,b</sup>	40.0 ± 6.7 <sup>a,b</sup>
6F-NDEA	8	0.16 ± 0.14 <sup>a</sup>	0.7 ± 0.7

<sup>a</sup>*p* < 0.05 versus saline  
<sup>c</sup>*p* < 0.05 versus NDELA

As shown in Table 1, quantitative analysis of the effects of NDELA, NEHEA, NDEA and 6F-NDEA on the development of foci and nodules was performed using GGT as a marker. NDEA, NDELA and NEHEA all induced the formation of focal lesions, NDEA being more potent than NDELA or NEHEA. 6F-NDEA did not induce a significant number of lesions.

Pretreatment with 26 μmol/kg 2,6-dichloro-4-nitrophenol, an inhibitor of sulfo-transferase (Sterzel & Eisenbrand, 1986), did not modify the development of GGT-positive lesions induced by NDEA but slightly inhibited initiating effects of NDELA and NEHEA

These results indicate that no simple correlation can be established between induction of SSB in DNA and the initiating capacity of NDELA, NEHEA and NDEA, since, even though the doses were equipotent in induction of SSB in DNA, the carcinogenic effects of the three nitrosamines were different. 6F-NDEA, a non-genotoxic analogue, had no initiating activity. Not only the genotoxicity of nitrosamines, but also DNA repair rate (Sterzel, 1986) and cell

necrosis (Ying *et al.*, 1981) might influence initiating capacity. It should also be pointed out that the lesions resulting from DNA SSB are not well characterized.

## REACTIONS OF *N*-NITROSOUREAS WITH CELL MEMBRANES

K. Yano<sup>1</sup>, M. Sonoda & Y. Sakagishi

*Departments of Chemistry and Biochemistry, Saitama Medical School,  
981 Kawakado, Moroyama-cho, Iruma-gun,  
Saitama 350-04, Japan*

The strongly carcinogenic *N*-nitrosoareas react with phosphatidylethanolamine (PE) in chicken erythrocyte ghosts or rat kidney cells, while weaker carcinogens do not. The reactions proceed through carbamylation of the amino group of PE by isocyanates generated from the agents. Many commercial isocyanates, including methyl isocyanate, react with PE similarly to the strong carcinogens, suggesting that this reaction may be involved in promotion in human cancer.

The development of cancer due to chemical carcinogens is assumed to be a multistep process involving initiation and promotion steps. According to this theory, ultimate carcinogens should have both initiating and promoting activities; however, the mechanism of their promoting action is unknown. We reported recently a positive correlation between the dynamic changes in chicken erythrocyte membranes induced by a series of five *N*-methyl-*N'*-aryl-*N*-nitrosoareas (X-Ar-NU) and their tumorigenic potencies (Yano *et al.*, 1985). In order to extend our previous findings, we have undertaken a study of the reactions of directly-acting carcinogens with cell membranes.

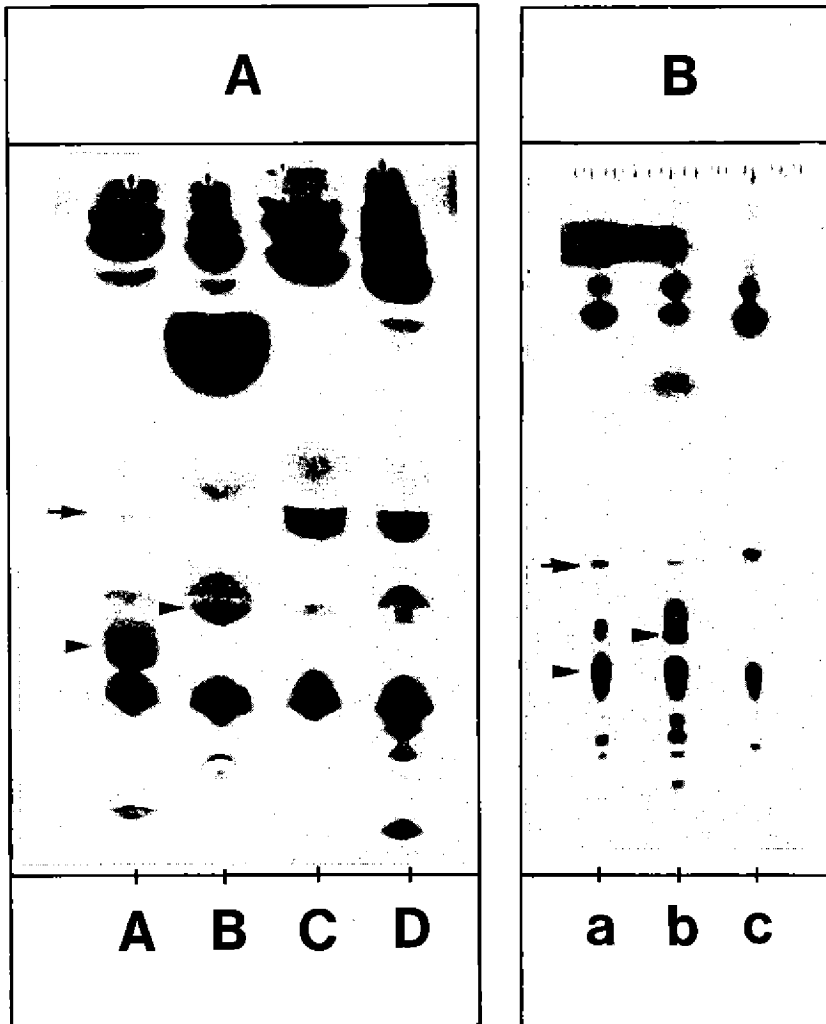
### Reactions of alkylating agents with cell membranes and phospholipids

We first reacted *N*-methyl-*N*-nitrosoareas (MNU), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and methyl methanesulfonate (MMS) with chicken erythrocyte ghosts, and analysed extracts of the reaction mixtures by thin-layer chromatography (Fig. 1A). The spot corresponding to PE almost disappeared in the presence of MNU or MNNG, which are highly potent carcinogens, but remained in the presence of MMS, which is a relatively weak carcinogen (Lawley, 1984). MNU and MNNG gave different spots, indicating that they react with PE by different mechanisms.

We carried out similar reactions using rat kidney cells instead of ghosts, to assess whether such reactions were involved in other cellular systems. The result obtained (Fig. 1B) prove the general validity of this hypothesis. To examine the possibility that carcinogens might react with other phospholipids in the membranes, we next studied the reactions of MNU with phosphatidic acid, phosphatidylcholine, phosphatidylinositol and phosphatidylserine. Comparisons of the thin-layer chromatographic analyses of the reaction mixtures with those of the corresponding blanks eliminated this possibility.

<sup>1</sup>To whom correspondence should be addressed

**Fig. 1. Thin-layer chromatograms of lipid extracts of reaction mixtures of alkylating agents with membranes: (A), chicken erythrocyte ghosts; (B), rat kidney cells**



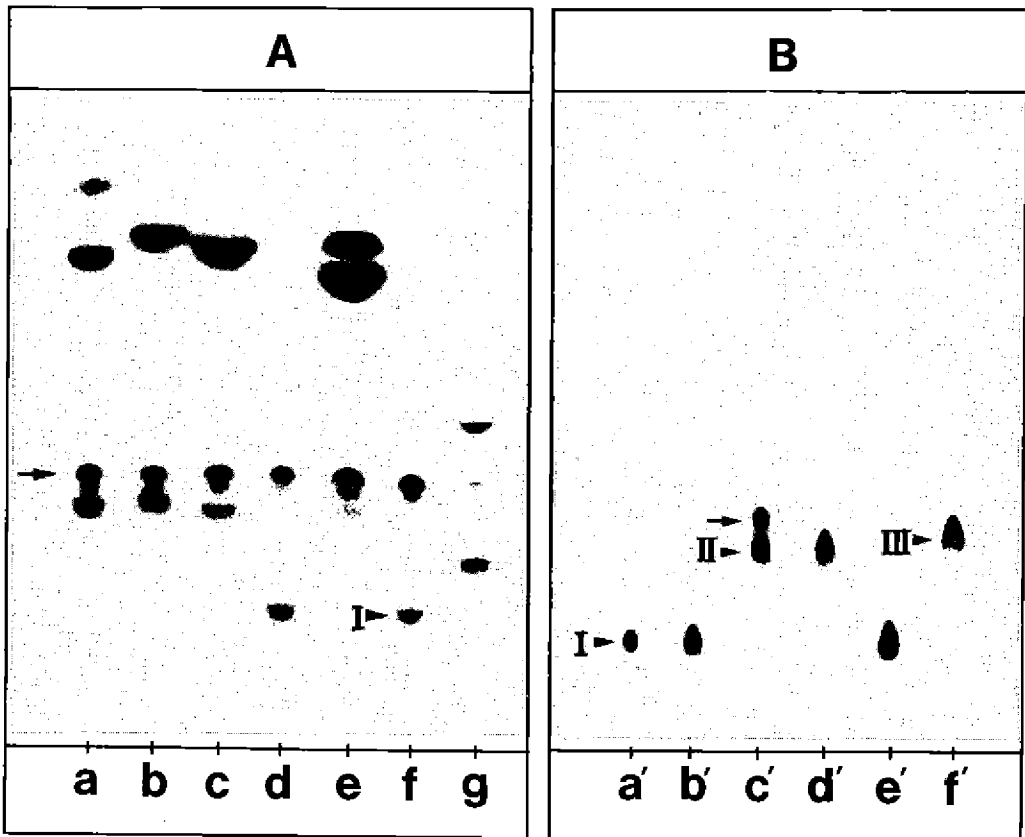
A, MNU; B, MNNG; C, MMS; D, dimethyl sulfoxide (DMSO); a, MNU; b, MNNG; c, MMS. Phosphatidylethanolamine (PE) is indicated by the arrow, and the reaction products are shown by the wedge-shaped characters. Chicken erythrocyte ghosts and rat kidney cells were prepared by the methods of Dodge *et al.* (1963) and of Jewell *et al.* (1975), respectively, just before they were used for the reactions. They were suspended in Hanks' buffer (approximately 35%), and 1 ml of each suspension was transferred into a tube. To each tube, cooled with ice, were added 200  $\mu\text{mol}$  of an alkylating agent (MNU, MNNG or MMS), 1 ml Hanks' buffer and 200  $\mu\text{l}$  DMSO. After sealing under nitrogen, the tubes were incubated at 37°C for 48 h with shaking. The extracted lipids were then dissolved in 100  $\mu\text{l}$   $\text{CHCl}_3$ . The  $\text{CHCl}_3$  solution (10  $\mu\text{l}$ ) was applied to a silica gel 60 thin-layer chromatographic plate, developed in a mixture of  $\text{CHCl}_3$ : $\text{CH}_3\text{OH}$ : $\text{H}_2\text{O}$  (65:25:4), and visualized with iodine vapour.

#### Relationship between reactivity of X-Ar-NU with PE and their tumorigenicity

Since a major target of MNU on cell membranes was shown to be PE, we further studied the reactions of X-Ar-NU (X =  $-\text{OCH}_3$ ,  $-\text{CH}_3$ ,  $-\text{H}$ ,  $-\text{Cl}$ ,  $-\text{COCH}_3$ ) and of MNU and

MNNG with commercial PE obtained from porcine liver. All X-Ar-NU compounds reacted to give a similar type of product ( $R_f = 0.356-0.358$ ), with the exception of Cl-Ar-NU, which produced an apparently different compound ( $R_f = 0.206$ ) (Fig. 2A). The tumorigenic potency of X-Ar-NU on mouse skin (percentage of mice with tumours) has been reported to be in the order H-Ar-NU (100),  $\text{CH}_3\text{O-Ar-NU}$  (80),  $\text{CH}_3\text{-Ar-NU}$  (72),  $\text{CH}_3\text{CO-Ar-NU}$  (29) and Cl-Ar-NU (0) (Yano *et al.*, 1984). Comparison of these data with the chromatographic results shows that Cl-Ar-NU, which is not a skin carcinogen, apparently does not react with PE in the same way as the other X-Ar-NU compounds. Moreover,  $\text{CH}_3\text{CO-Ar-NU}$ , which is a much less potent carcinogen than H-Ar-NU,  $\text{CH}_3\text{O-Ar-NU}$  or  $\text{CH}_3\text{-Ar-NU}$ , appears to be the least reactive compound among them.

**Fig. 2. Thin-layer chromatograms of: A, extracts of the reaction mixtures of X-Ar-NU, MNU and MNNG with PE; B, separated products (except lane c') of the reaction mixtures of nitrosoureas or isocyanates with PE**



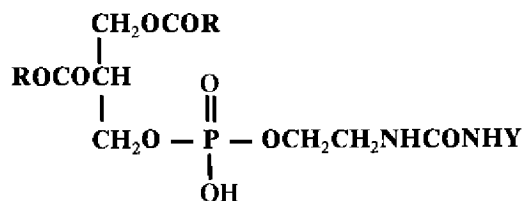
a,  $\text{CH}_3\text{O-Ar-NU} + \text{PE}$ ; b,  $\text{CH}_3\text{-Ar-NU} + \text{PE}$ ; c, H-Ar-NU + PE; d, Cl-Ar-NU + PE; e,  $\text{CH}_3\text{CO-Ar-NU} + \text{PE}$ ; f, MNU + PE; g, MNNG + PE; a', MNU + PE; b', HNCN + PE; c', H-Ar-NU + PE; d',  $\text{C}_6\text{H}_5\text{NCO} + \text{PE}$ ; e', Cl-Ar-NU + PE; f',  $\text{ClC}_6\text{H}_4\text{NCO} + \text{PE}$

**Carbamoylation of PE by nitrosoureas and isocyanates**

We determined the chemical structure of the product (I) obtained from the reaction of MNU with PE by spectrometric methods (Figs 2B, 3), and confirmed our assignment by synthesizing I from a reaction of isocyanic acid with PE. To generalize the above carbamoylation, we chose H-Ar-NU (the most potent carcinogen among X-Ar-NU) and Cl-Ar-NU (not a carcinogen) as well as phenylisocyanate ( $C_6H_5NCO$ ) and *para*-chlorophenylisocyanate ( $ClC_6H_4NCO$ ) and carried out similar reactions: H-Ar-NU and  $C_6H_5NCO$  gave the same product (II), while Cl-Ar-NU and  $ClC_6H_4NCO$  gave I and III, respectively. These findings suggest that the reaction mechanism of Cl-Ar-NU is different from that of other X-Ar-NU, and that this difference may be related to its lack of tumorigenic activity.

These results communicate an urgent message: since the Bhopal incident occurred in December 1984, methyl isocyanate has attracted wide attention with regard to its possible carcinogenic effects. Until now, its possible carcinogenicity has not been investigated; our results suggest that isocyanates, including methyl isocyanate (data not shown), which are widely used as chemical intermediates for polymers and pharmaceuticals, can react with PE in cell membranes just as strong carcinogens do. Such reactions probably play an important role in the multistep process of tumour promotion, since they change not only the lipid composition but also the polar head group composition of membranes, both of which regulate many important functions of the cell membranes (Roos & Choppin, 1984; Schroeder & Gardiner, 1984; Yechiel & Barenholz, 1985).

**Fig. 3. Chemical structures of the products (I, II and III) of reactions of nitrosoureas and isocyanates with PE**



Y : — H (I); —  $C_6H_5$  (II); —  $C_6H_4Cl$  (III)

**Acknowledgements**

We thank Professor K. Uyemura and Mr Y. Sakamoto for measurements of the mass spectra. We also thank Dr K. Matsushita of JEOL, and Dr T. Endo and Dr T. Yokozawa, Tokyo Institute of Technology, for measurements of the nuclear magnetic resonance and infrared spectra.



## **METHODS FOR DETECTION**

## AN IMPROVED METHOD FOR ANALYSIS OF TOTAL N-NITROSO COMPOUNDS IN GASTRIC JUICE

B. Pignatelli, I. Richard, M.-C. Bourgade & H. Bartsch

*International Agency for Research on Cancer  
150, cours Albert Thomas, Lyon, France*

An improved procedure for the analysis of total *N*-nitroso compounds (NOC) in human gastric juice was developed by modifying previous methods. The gastric juice sample, treated with sulfamic acid to remove nitrite, is injected directly into refluxing ethyl acetate containing either acetic acid for determining thermo/acetic acid-labile-thermal energy analyser (TEA)-responsive compounds (TAC), or into hydrogen bromide for the analysis of TAC and NOC. The nitric oxide (NO) levels released are measured by chemiluminescence by TEA, and the difference between the two determinations represents the concentrations of NOC in gastric juice. This method also allows the determination of nitrite and is not affected by nitrate concentrations up to 1000  $\mu\text{mol/l}$ . The method was found to be reproducible and sensitive (detection limit, 0.02  $\mu\text{mol NOC/l}$ ), requiring only small volumes of gastric juice and no prior extraction. Because the difficulties arising from the 'system response' to the denitrosating agent and variability of NO release by acetic acid from nitrite were eliminated, this improved method can more accurately distinguish NOC from most other TEA-responsive species. Suitable techniques for stabilizing gastric juice samples from duodenal ulcer/atrophic gastritis patients and the influence of the time and storage conditions on NOC concentrations have been studied.

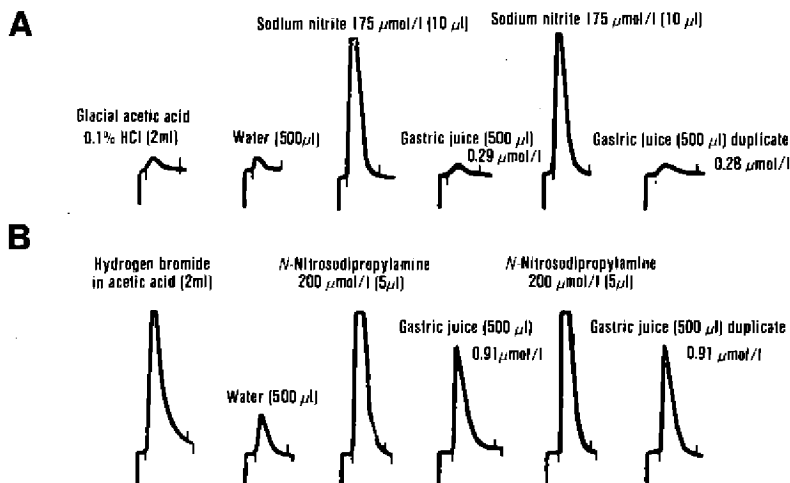
Carcinogenic NOC formed in the stomach have been suggested as causative agents in the pathogenesis of gastric cancer (Correa *et al.*, 1975; Mirvish, 1983). However, recent applications of the two available methods for group determination of total NOC (Walters *et al.*, 1978; Bavin *et al.*, 1982; Walters *et al.*, 1983) gave rise to contradictory findings, and the reported concentrations of NOC in human gastric juice showed large interlaboratory variations (Ruddell *et al.*, 1978; Reed *et al.*, 1981a; Milton-Thompson *et al.*, 1982; Walters *et al.*, 1982; Dang Vu *et al.*, 1983; Bartsch *et al.*, 1984; Keighley *et al.*, 1984; Kyrtopoulos *et al.*, 1985; Hall *et al.*, 1986). Both methods have been subject to criticism (Bavin *et al.*, 1982; Smith *et al.*, 1983; Walters *et al.*, 1984).

The group-selective procedure for the determination of total NOC (Walters *et al.*, 1978, 1983) is based on their chemical denitrosation with hydrogen bromide and chemiluminescence detection of the released NO. Sequential addition of acetic acid and hydrogen bromide allows distinction between NOC and most of the other potentially NO-releasing substances. Because this technique has some limitations, modified procedures have been proposed (Bavin *et al.*, 1982, Dang Vu *et al.*, 1983). Although they permit the direct analysis of aqueous samples, other interfering NO-releasing compounds cannot be distinguished from NOC. Our aim was to develop a more reliable procedure for analysing total NOC in human gastric juice.

### Method of analysis for total NOC

Operating conditions, glassware assembly and reagents were the same as described by Walters *et al.* (1983), except for three modifications: (i) a gas stream filter (CTR<sup>TM</sup>, Thermo Electron) was added between the last cold trap and the TEA input in order to improve the selective passage of NO; (ii) in the assembly for mode A analysis (see below), a dry caustic soda trap replaced the three successive traps containing 6 N sodium hydroxide used for mode B analysis; (iii) because of the variable TEA response of the nitrite standard (observed after its decomposition by acetic acid alone), 0.1% hydrochloric acid was added, which led to excellent reproducibility and sensitivity without further decomposition of NOC (Fig. 1A). The chemiluminescence detectors of NO were TEA models 502 (mode B) and 543 (mode A) (Thermo Electron Corp., Waltham, MA, USA), connected to integrators (Hewlett Packard 3390 A model).

**Fig. 1. Analysis of gastric juice for total NOC; A, TAC; B, TAC + NOC**



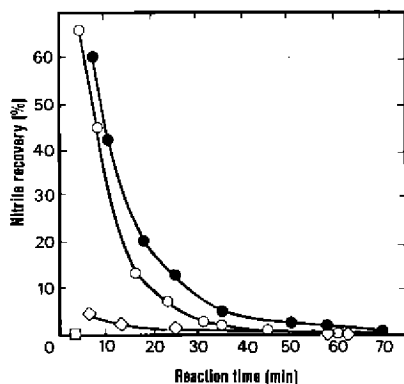
A typical example of the analysis of gastric juice for total NOC is shown in Figure 1. The gastric juice sample was treated with sulfamic acid (2% w/v, pH < 2) to destroy nitrite and analysed by two parallel procedures: (1) mode A: gastric juice sample injected directly into refluxing ethyl acetate containing acetic acid/0.1% hydrochloric acid, and the TAC measured; (2) mode B: injected directly into refluxing ethyl acetate containing hydrogen bromide (15% in glacial acetic acid), and both TAC and NOC determined simultaneously. The difference between the two modes (B-A) represents the concentration of NOC in gastric juice. The NO released was measured by chemiluminescence with TEA. The concentration of nitrite in gastric juice can also be determined by the difference between TEA measurements following injection of gastric juice with and without sulfamic acid treatment according to procedure A. It was noted that the first injection of water (500 µl) gave a variable detector response (Fig. 1), but subsequent injections did not give a similar response at any stage in either analytical procedure. Concentrations of TAC and TAC plus NOC were calculated from the ratio of the peak area of the sample to that of a standard sodium nitrite

solution (Fig. 1A) and a standard *N*-nitrosodipropyl amine (NDPA) solution (Fig. 1B), respectively. Equimolar solutions of nitrite and NDPA led to identical TEA responses after denitrosation by hydrogen bromide. Therefore, to estimate the concentration of NOC, the amount of TAC calculated from nitrite as external standard (Fig. 1A) can be subtracted from the TEA response arising from denitrosation by hydrogen bromide (Fig. 1B). Both standard solutions gave reproducible values for the peak areas, with a coefficient of variation of 2%. The presence of water in ethyl acetate (3–5% v/v) or of sodium nitrate up to 1000  $\mu\text{mol/l}$ , with or without treatment by sulfamic acid, and up to one month's storage at  $-20^\circ\text{C}$ , did not affect the analysis. Higher concentrations of nitrate, although never found in gastric juice samples, led to a false positive TEA response.

### Conditions to prevent artefactual nitrosation

Three known inhibitors of *N*-nitrosation, hydrazine sulfate, ascorbic acid and sulfamic acid, were compared for their efficiency as nitrite scavengers. Aqueous sodium nitrite solutions at concentration ranging from 20 to 400  $\mu\text{mol/l}$  were treated as described in the legend to Figure 2. The remaining nitrite in the aqueous solution was measured by TEA as a function of reaction time (Fig. 2). Sulfamic acid was the most effective agent for removal of nitrite at  $\text{pH} < 2$ . At  $\text{pH} 4$ , hydrazine sulfate did not react with nitrite as quickly as did sulfamic acid; however, when a hydrazine sulfate solution (see legend to Fig. 2) with a  $\text{pH}$  of 1.48 was not adjusted to  $\text{pH} 4$  prior to addition to nitrite solution, the  $\text{pH}$  fell to 2 and at least 2–3 min were necessary to establish  $\text{pH} 4$ . At  $\text{pH} < 2$ , hydrazine sulfate was as efficient as sulfamic acid in removing nitrite. The above observations could explain why hydrazine sulfate has been reported to be an efficient nitrite trap at  $\text{pH} 4$  (Bavin *et al.*, 1982).

**Fig. 2. Comparison of the efficiency of three nitrite scavengers**



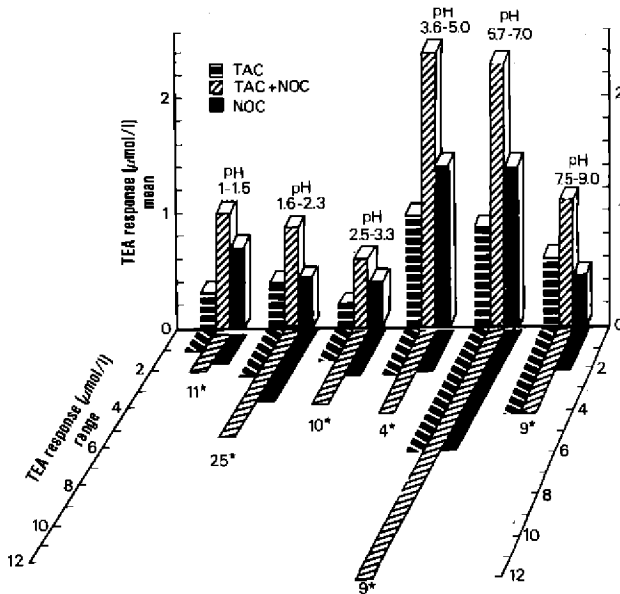
●, 0.3 ml per ml of a saturated aqueous hydrazine sulfate solution (3.4 g/100 ml water) adjusted to  $\text{pH} 4$ , the final  $\text{pH}$  being adjusted to 4; ○, 0.3 ml per ml of a saturated aqueous hydrazine sulfate solution (3.4 g/100 ml water) adjusted to  $\text{pH} 4$  and containing phthalate (10 mg per ml), the final  $\text{pH}$  being adjusted to 4; ◇, 10 mg/ml ascorbic acid, to a final  $\text{pH}$  of 2.9; □, 20 mg/ml sulfamic acid, to a final  $\text{pH}$  of 1. Sodium nitrite concentration, 43.4  $\mu\text{mol/l}$

After treatment of a nitrite solution (220  $\mu\text{mol/l}$ ) with sulfamic acid (2% w/v) and storage at  $-20^\circ\text{C}$  for up to six weeks, no nitrite was detectable. When sodium nitrite (final concentration, 229  $\mu\text{mol/l}$ ) was reacted with 2,6-dimethylmorpholine (final concentration, 162  $\mu\text{mol/l}$ ) containing sulfamic acid, no nitrosamine was detectable after 5 min of reaction and after storage at  $-20^\circ\text{C}$  for up to one month.

### Analysis of gastric juice samples for total NOC

A total of 68 gastric juice samples, obtained from patients before and after operation for duodenal ulcer ( $n = 59$ ) or with chronic atrophic gastritis ( $n = 9$ ) were analysed for NOC. Because of small sample volumes, some gastric juices with similar  $\text{pH}$  were pooled. The results (Fig. 3) are discussed below. To verify the suitability of this analytical method, (i) the efficiency of sulfamic acid and hydrazine sulfate as nitrite scavengers was investigated in nitrite-spiked gastric juice samples; (ii) the stability of NOC following these two treatments and the influence of time and storage conditions on NOC concentrations were examined; and (iii) artefactual *N*-nitrosation was verified after spiking gastric juice samples with 2,6-dimethylmorpholine.

**Fig. 3. Concentrations of TAC and total NOC in gastric juice samples as a function of pH; \*, number of samples analysed**



After collection, gastric juices were frozen and analysed within three days. After thawing, they were vigorously homogenized (Vortex) and, when necessary, centrifuged. Samples were then divided into two — one treated with sulfamic acid and the other with hydrazine sulfate, under the conditions described in the legend to Figure 2. For each sample, these two treatments were done in parallel. Aliquots were subsequently transferred in glass/Teflon-joint, stoppered tubes to allow spiking with sodium nitrite (200-230  $\mu\text{mol/l}$ ) or with 2,6-dimethylmorpholine (130-150  $\mu\text{mol/l}$ ) or with standard NOC (6-25  $\mu\text{mol/l}$ ). Multiple preparations were carried out for each assay condition. Aliquots not analysed at once were immediately frozen at  $-20^\circ\text{C}$ .

For the analysis of gastric juice samples treated with sulfamic acid, the coefficients of variation from duplicate/triplicate TEA measurements (Fig. 1) were 5-10%. The limit of detection was 0.02  $\mu\text{mol/l}$ . When gastric juice samples were treated with hydrazine sulfate, the same analytical procedure led to broader and more tailing peaks, especially for NO released by TAC. In addition, the TEA response of standard nitrite (Fig. 1A) was reduced up to 50% after the first sample injection, and the response did not improve after addition of more acid. As a result, the latter measurement was less accurate and duplicates could not be analysed afterwards. After stabilization of gastric juice samples with sulfamic acid, the addition to the samples of nitrite ( $n = 27$ ) or 2,6-dimethylmorpholine ( $n = 20$ ) did not produce any artefactual nitrosation. After treatment by hydrazine sulfate at pH 4, no additional nitrosation of added 2,6-dimethylmorpholine was found ( $n = 8$ ), but artefacts (i.e., remaining nitrite and formed NOC) were observed in all samples ( $n = 20$ ).

The stability of aqueous solutions of nine reference NOC (13-26  $\mu\text{mol/l}$ ) was measured following treatment by sulfamic acid or hydrazine sulfate, after storage at  $+4^\circ\text{C}$  for 1 h or at  $-20^\circ\text{C}$  for one month. The stabilities under these storage conditions and after both treatments decreased similarly in the following order (recoveries 60 - 96%): *N*-nitrosoproline  $\approx$  *N*-nitrosodiethylamine  $\approx$  *N*-nitrosopentylmethylamine  $\approx$  *N*-nitrosodiethanolamine  $>$  *N*-propyl-*N'*-nitro-*N*-nitrosoguanidine  $\approx$  *N*-methyl-*N*-nitrosourea (MNU)  $\gg$  *N*-propyl-*N*-nitrosourethane  $>$  *N*-methyl-*N*-nitrosourethane (MNUT). As an exception (after one month's storage at  $-20^\circ\text{C}$ ), 90% of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) was recovered following sulfamic acid treatment, but none after hydrazine sulfate treatment. It

was noted that the stability of homologues of *N*-nitroso guanidines/urethanes increased with increasing alkyl chain length, as observed by Haga *et al.* (1972). Compounds that are more susceptible to decomposition (MNU, MNUT, MNNG) and *N*-nitrosodiethylamine, *N*-nitrosopentylmethylamine and *N*-nitrosoproline were selected for further studies in stabilized gastric juice samples (Table 1) to which each of these compounds have been added.

**Table 1. Recovery of NOC in gastric juice as a function of storage time at  $-20^{\circ}\text{C}$**

NOC <sup>a</sup> added to gastric juice ( $\mu\text{mol/L}$ )	pH range	Number of samples	Treatment <sup>b</sup>	% Recovery of NOC <sup>c</sup> (mean $\pm$ SD)		
				Days of storage at $-20^{\circ}\text{C}$		
				0-1	15-19	30-34
None	3.3-8.9	9	SA	-	69.9 $\pm$ 24.6	61.2 $\pm$ 28.2
	1.2-3.0	18	SA	-	67.7 $\pm$ 29.9	61.2 $\pm$ 33.9
NPRO (14)	1.5-5.9	3	SA	99.0 $\pm$ 1.0	96.3 $\pm$ 1.5	94.3 $\pm$ 1.5
NA (12-16.4)	1.6-6.3	5	SA	97.8 $\pm$ 2.2	92.4 $\pm$ 3.9	91.2 $\pm$ 2.9
MNNG (11-14.4)	6.7-7.9	4	SA	94.7 $\pm$ 6.7	73.5 $\pm$ 5.2	57.0 $\pm$ 6.3
			HS	88.7 $\pm$ 13.8	28.5 $\pm$ 18.0	15.5 $\pm$ 15.9
	1.5-1.7	3	SA	87.7 $\pm$ 3.8	21.7 $\pm$ 13.2	8.3 $\pm$ 6.8
			HS	98.3 $\pm$ 2.9	11.0 $\pm$ 3.5	8.0 $\pm$ 6.1
MNU (12.7-22)	3.3-5.9	3	SA	99.0 $\pm$ 1.4	95.0 $\pm$ 7.1	91.5 $\pm$ 2.1
			HS	90.5 $\pm$ 13.4	86.5 $\pm$ 7.8	83.0 $\pm$ 2.8
	1.2-2.1	5	SA	89.6 $\pm$ 6.9	40.8 $\pm$ 27.7	22.8 $\pm$ 25.3
			HS	96.0 $\pm$ 5.8	86.6 $\pm$ 5.8	69.6 $\pm$ 14.0
MNUT (6-24.7)	6.3-7.5	3	SA	83.5 $\pm$ 0.7	67.5 $\pm$ 7.8	57.0 $\pm$ 1.4
			HS	94.0 $\pm$ 1.4	85.0 $\pm$ 5.7	61.5 $\pm$ 19.1
	1.2-3.0	(10)	SA	67.2 $\pm$ 21.6	15.0 $\pm$ 17.1	13.2 $\pm$ 14.1
			SA*	48.7 $\pm$ 12.0	42.0 $\pm$ 10.2	41.5 $\pm$ 10.5
			HS	91.7 $\pm$ 5.6	81.2 $\pm$ 11.8	61.6 $\pm$ 31.8

<sup>a</sup>NPRO, *N*-nitrosoproline; NA, *N*-nitrosodiethylamine or *N*-nitrosopentylmethylamine; the NOC solutions were freshly prepared and their concentrations were checked.

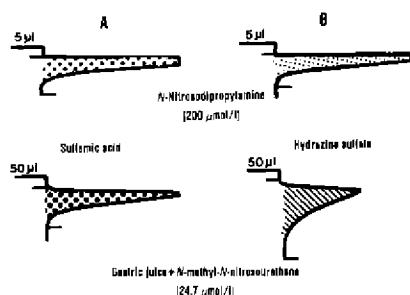
<sup>b</sup>SA, sulfamic acid, 2% w/v, pH 1; SA\*, sulfamic acid, 2% w/v, pH 1 during 5-10 min, then adjustment to pH 4; HS, hydrazine sulfate, 0.3 ml of 3.4% w/v solution per ml, pH 4

<sup>c</sup>Calculated taking as 100% the measurement of NOC concentration in 2 ml water spiked with 10  $\mu\text{l}$  of freshly prepared NOC solution (to simulate a gastric juice sample). The amount of NOC measured in each unspiked sample was subtracted from values obtained with the spiked sample after the same storage time. For unspiked gastric juice, the first determination of NOC was taken as 100%.

When reference NOC were added to gastric juice and analysed within 24 h of storage at  $-20^{\circ}\text{C}$ , all were recovered at 70-99%. In gastric juice samples that were treated with hydrazine sulfate, MNU and MNUT were rather stable, but MNNG was

largely decomposed after storage for 15 days at  $-20^{\circ}\text{C}$ . After treatment with sulfamic acid, the TEA peaks were sharper (Fig. 4), and MNNG, MNU and MNUT were reasonably stable in gastric juice samples that had initially a neutral/basic pH. However, MNU was much less stable and MNNG/MNUT were quite unstable in samples with an initial acidic pH. The adjustment to pH 4 after sulfamic acid treatment and before storage prolonged the stability of MNUT. Remarkably, the unidentified NOC measured as a group in our gastric juice samples ( $n = 27$ ) treated with sulfamic acid were less stable than nitrosoaminoacids/-nitrosamines but more stable than *N*-nitrosooureas/*N*-nitrosoourethanes when stored at  $-20^{\circ}\text{C}$  for up to one month. Their recoveries were not dependent on the initial pH of gastric juice samples.

**Fig. 4.** TEA response after denitrosation by hydrogen bromide of a gastric juice spiked with MNUT after treatment by sulfamic acid (A) or by hydrazine sulfate (B)



Means and ranges of TEA responses for the calculated TAC, (TAC plus NOC) and NOC concentrations in the gastric juice samples analysed (stabilized by sulfamic acid) are plotted for several pH ranges (Fig. 3). Although the sample size was small, certain conclusions can be drawn: (i) the concentrations (means) of NOC were not significantly different in gastric juice samples with extreme acidic or basic pH (0.5 versus 0.6  $\mu\text{mol/l}$ , respectively); (ii) in contrast, samples at pH 3.6 to 7.0 had higher (mean) NOC concentrations (1.4  $\mu\text{mol/l}$ ); (iii) the concentrations of TAC were as pH-dependent as the NOC; (iv) the TAC concentrations profoundly influenced total TEA-responsive compounds since the ratio [NOC]:[TAC] varied in individual samples from 0.2 to 5. One study suggests that sulfamic acid may enhance nitrosamine formation above pH 4 (Ziebarth & Teichmann, 1980).

Artefactual formation of NOC was not observed after spiking gastric juice samples at an initial pH of  $\geq 4$  with nitrite and 2,6-dimethylmorpholine and treating them with sulfamic acid at pH  $\leq 2$ . We therefore exclude catalysis of nitrosation by sulfamic acid as a factor in the pH profile observed for NOC in their gastric juice samples.

A progressive increase of NOC levels in gastric juice with rise of pH has been reported (Ruddell *et al.*, 1978; Reed *et al.*, 1981a; Walters *et al.*, 1982). These results support the hypothesis according to which gastric hypochlorhydria permits bacterial overgrowth leading to increased nitrite formation, NOC formation and, hence, an elevated risk of gastric cancer. Other studies, however, failed to show any significant variation between levels of NOC in gastric juice and pH (Bartsch *et al.*, 1984; Kyrtopoulos *et al.*, 1985). Moreover, much lower concentrations of total NOC in hypoacidic as compared to acidic gastric juices were obtained using the methodology of Bavin *et al.* (1982) (Milton-Thomson *et al.*, 1982; Keighley *et al.*, 1984; Hall *et al.*, 1986). In these latter studies, the absolute values were up to five times greater than those measured by the method of Walters *et al.* (1978, 1983). In our study, intermediate values for NOC concentration (mean and ranges) were obtained that were closer to those reported by users of Walters' method. The latter procedure requires the extraction of NOC in ethyl acetate (probably with poor yields of some NOC), and the acidic pH ( $< 2$ ) precludes the extraction of basic NOC from gastric juice. The method of Bavin *et al.* (1982) may overestimate NOC in gastric

juice, because TAC are not distinguished from NOC (Table 2). The existence in the 68 gastric juice samples of an optimal NOC concentration at pH 3.6-7.0 (Fig. 3) may result from two counteracting pH-dependent variables: (i) the available nitrite concentration that increases with rising pH, i.e., because of higher numbers of nitrate-reducing bacteria in the stomach of achlorohydric subjects and increased nitrite stability; (ii) the rate of acid-catalysed nitrosation that decreases with elevated pH.

**Table 2. Summary of results which show the extent to which TAC influence the determination of TEA-responsive compounds if they are measured after denitrosation by hydrogen bromide only**

pH range	% Samples containing TAC (no. of samples)	Effect of TAC on measurement of NOC	
		TAC ( $\mu\text{mol/l}$ )	Relative $\frac{\text{TAC} + \text{NOC}}{\text{NOC}}$
1-1.5	55 (6)	0-1.2	1-4
1.6-2.3	36 (9)	0-2.2	1-6.5
2.5-3.3	50 (5)	0-1.5	1-2.4
3.6-5.0	50 (2)	0-2.2	1-1.2
5.7-7.0	56 (5)	0-6.4	1-8
7.5-9.0	56 (5)	0-4.0	1-2

under the same conditions as NOC (Walters *et al.*, 1982; Smith *et al.*, 1983; Walters *et al.*, 1984). Our improved method for analysing total NOC in gastric juice maintains a great selectivity and offers additional advantages. It is hoped that its application can resolve current controversies (see Introduction) concerning the role of NOC in gastric cancer.

#### Acknowledgements

The authors wish to thank Dr Moulinier, Dr Y. Minaire, Dr J. Forichon and their medical staff at Edouard-Herriot Hospital, Lyon, France, for providing gastric juice samples.

In conclusion, our findings (Table 2) in general confirm the criticism made by Smith *et al.* (1983) about modified versions (Bavin *et al.*, 1982; Dang Vu *et al.*, 1983) of Walters' procedure (Walters *et al.*, 1978, 1983). The magnitude of the false-positive response from heat-labile (e.g., pseudonitrosites, nitrosothiols) and acid-sensitive compounds (e.g., alkyl nitrites) can be very important. In addition, a false-positive response may arise from the very first injection of gastric juice without prior injection of water (Fig. 1). In our method, only nitrolic acids, nitrothiols and some aliphatic C-nitroso compounds can give a response



# THERMAL ENERGY ANALYSIS OF N-NITROSO COMPOUNDS, INCLUDING UREAS, URETHANES AND GUANIDINES

D.H. Fine, E.U. Goff, R.A. Johnson, Jr & D.P. Rounbehler

*Thermedics Inc., 470 Wildwood Street, Woburn, MA 01888, USA*

**Simple, selective, sensitive analytical methods have been developed for a number of *N*-nitroso compounds (NOC) which are applicable to environmental and biological matrices and have been used to quantify nitrosourea anticancer drugs in human plasma. These new chromatographic detection windows can be used to screen suspect matrices for NOC.**

The lack of adequate analytical techniques for some NOC (*N*-nitrosoureas, *N*-nitrosourethanes, *N*-nitrosamides, *N*-nitrosoguanidines, *N*-nitrososulfonamides) that are both sensitive enough for quantification at ppb levels and selective enough for routine application to environmental and biological matrices has limited understanding of the significance of these compounds. Efforts have been directed to developing techniques for assessing human exposure to nitrosamides.

## **Relevance of nitrosamides**

Despite the presence of precursors and facile kinetics (e.g., Mirvish, 1975), only nitrosamines have been identified in the environment as a result of unintentional nitrosation. Some analytical data indicate relatively high total NOC contents in some matrices (e.g., Bavin *et al.*, 1982). A correlation has been observed between ingested nitrite level and cancer incidence, and it has been postulated that NOC may be partly responsible (e.g., Endo *et al.*, 1977). In addition, some nitrosoureas are used for clinical treatment of cancer. Interindividual variations in drug pharmacokinetics necessitate monitoring of their levels in plasma (Weinkam & Lin, 1982).

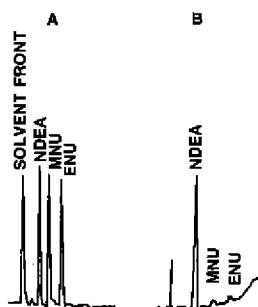
## **Development of detection techniques**

The Thermal Energy Analyzer (TEA) has been modified [TEA(amide)] to make possible analytical methods for many NOC similar to those now available for nitrosamines. The detection limits and selectivity of the gas chromatograph (GC)-TEA(amide) are comparable to those of the standard TEA (Johnson *et al.*, 1986a). The standard denitrosation response has been found unreliable for such analyses (Fig. 1). An amide detector, based on high-performance liquid chromatography-TEA(amide) has been previously reported (Fine *et al.*, 1983; Goff & Fine, unpublished data). This detector has been used for the detection of *N*-nitrosourea anticancer drugs.

## **Development of analytical methods**

The advantages of capillary over packed GC columns have been described (e.g., Jennings & Mehran, 1986). NOC that have been analysed by this method include MNU,

**Fig. 1. (A) Response of some representative compounds in the GC-TEA (amide); (B) response of same compounds with standard TEA**

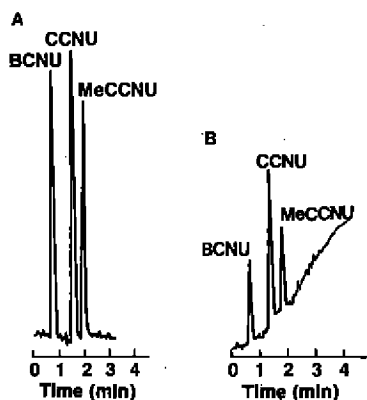


NDEA, *N*-nitrosodiethylamine; MNU, *N*-methyl-*N*-nitrosoarene; ENU, *N*-ethyl-*N*-nitrosoarene. Column, DB-1, 27 m  $\times$  0.53 mm; film, 1.5  $\mu$ m; carrier, He  $\sim$  5 psi head pressure; oven, 80-110°C at 5°C/min; injection, 1  $\mu$ l cool on-column (100°C)

ENU and *N*-methyl-*N*-nitrosoarene (Fine *et al.*, 1983; Issenberg, personal communication). The technique has been extended to the analysis of other NOC, including *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, *N*-nitrosodiethanolamine, 1,3-bis(*N*-chloroethyl)-1-nitrosoarene, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosoarene and 1-(2-chloroethyl)-3-(4-methyl)cyclohexyl-1-nitrosoarene. Less than 1 pmol NOC can be chromatographed and detected (Fig. 2). Retention times are indicative of molecular weight and structure. These compounds, which have been considered nonvolatile, are readily analysed by capillary GC. Use of cool, on-column injection and a retention gap injection port enabled injections containing nonvolatile compounds to be made without washing the analytical column.

A method for determination of nitrosoarene drugs has been developed at detection limits in human plasma of about 1 ppb (Johnson *et al.*, 1986b). The procedure involves extraction, evaporation of solvent and reconstitution followed by chromatographic separation and detection with a modified TEA. More than 90% recovery of fortified drugs is observed, and no peak other than the compounds of interest was detected. Analysis (extraction, chromatography and detection) was complete in 10-15 min. The biological half-lives were similar to those observed with other methods (Johnson *et al.*, 1986b).

**Fig. 2. (A) Injection of 1 pmol each of the nitrosoarene anticancer drugs 1,3-bis(2-chloroethyl)-1-nitrosoarene (BCNU), 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosoarene (CCNU) and 1-(2-chloroethyl)-3-(4-methyl)cyclohexyl-1-nitrosoarene (MeCCNU); (B) analysis of  $\sim$  20 ppb of the drugs spiked in plasma**



Column, DB-5, 0.5 m  $\times$  0.53 mm; film, 1.5  $\mu$ m; carrier, hydrogen at 0.6 torr TEA vacuum; oven, 85-110°C at 5°C/min; injection, 1  $\mu$ l cool on-column with retention gap (100°C); detector, GC-TEA (amide)

Although nitrosamides are less stable than nitrosamines, many can be determined without difficulty. Methods are being developed for a variety of matrices, including air and bulk samples, cosmetics, drugs, foodstuffs, tobacco, urine and gastric juice. The general usefulness of the techniques has been demonstrated, and an assessment of human exposure to a number of NOC not previously routinely analysed at ppb levels is under way.

**Acknowledgement**

We gratefully acknowledge the contributions of J. Buckley, Dr K. Dasse, J. Stevens, M. Weber and Dr G. Wendel of Thermedics and also that of Dr G. Edwards of Toxicon Associates. This investigation was supported by PHS Grant Number 5 R01 CA42586-02 awarded by the National Cancer Institute, DHHS, USA.

## AN INVESTIGATION OF APPARENT TOTAL N-NITROSO COMPOUNDS IN BEER

R.C. Massey<sup>1</sup>, P.E. Key<sup>1</sup>, D.J. McWeeny<sup>1</sup> & M.E. Knowles<sup>2</sup>

<sup>1</sup>Ministry of Agriculture, Fisheries and Food,  
Food Science Laboratory, Queen Street, Norwich NR2 4SX; and

<sup>2</sup>Ministry of Agriculture, Fisheries and Food,  
Great Westminster House, Horseferry Road, London SW1P 2AE, UK

The concentration of apparent total *N*-nitroso compounds (ATNC) in beer has been investigated using a group-selective procedure based on chemical denitrosation with hydrogen bromide and chemiluminescence detection of the released nitric oxide. In a survey of samples of 40 brands of beer and lager, detectable levels of ATNC were present in 17 samples at concentrations of 20-100  $\mu\text{g N-NO/kg}$  in 11 and 100-500  $\mu\text{g N-NO/kg}$  in six. To determine the origin of ATNC in beer the production of a commercial batch was examined in detail. ATNC levels were below the detection limit in the sweet wort (aqueous extract of malt), bitter wort (malt extract boiled with hops) and also at the start of fermentation, but during the course of fermentation the concentration of ATNC increased appreciably and that of inorganic nitrate decreased; detectable, though transitory, levels of inorganic nitrite were observed. None of the brewing ingredients contained sufficiently high enough levels of ATNC to account for the concentration of these compounds present in the beer after fermentation. These findings suggest that the presence of detectable levels of ATNC in some beers is a result of *N*-nitrosation reactions occurring in the fermenting wort with the nitrosating species derived from reduction of nitrate, due probably to the presence of microbial species with nitrate reductase activity.

The occurrence of trace levels of the volatile *N*-nitrosamine *N*-nitrosodimethylamine (NDMA) in beer is now well established (Spiegelhalter *et al.*, 1980a). Much less is known about the possible incidence of nonvolatile *N*-nitroso compounds, although recent reports (Massey *et al.*, 1982, 1984a) suggest that higher levels may be present in some beers. This paper describes an investigation into the occurrence of ATNC in beer and the factors that affect the formation of these compounds.

### Occurrence of ATNC in beer

In a survey of retail samples of 40 brands of beer and lager, the ATNC concentration was below the 20  $\mu\text{g N-NO/kg}$  limit of detection in 23 samples. Detectable levels of ATNC were present in the remaining samples, 11 having concentrations of 20-100  $\mu\text{g N-NO/kg}$ , and six, 100-500  $\mu\text{g N-NO/kg}$ .

A previous survey in our laboratory (unpublished data) of over 50 commercial samples showed that the ATNC levels in malt are not sufficiently high to produce levels of ATNC in excess of 20  $\mu\text{g N-NO/kg}$  in finished beer. This suggests that malt may not be the major source of ATNC in beer, in contrast to the situation with NDMA. To investigate this possibility the production of a commercial batch of beer was examined.

### Formation of ATNC during brewing

Brewing ingredients and process samples taken at various stages of production of a commercial batch of beer were analysed for ATNC, nitrite and nitrate. The results are shown in Table 1. Concentrations of ATNC in both the sweet wort and boiled wort were below the 20  $\mu\text{g N-NO/kg}$  detection limit but increased markedly to 140  $\mu\text{g N-NO/kg}$  during the first 12 h of fermentation. The concentration decreased to 100-110  $\mu\text{g N-NO/kg}$  on racking at completion of fermentation, and subsequent bottling and pasteurization processes had no effect on the level of ATNC.

**Table 1. Levels of ATNC, nitrate and nitrite in brewing ingredients and process samples during production of a batch of commercial beer<sup>a</sup>**

Sample	ATNC ( $\mu\text{g N-NO/kg}$ )	Nitrate ( $\text{mg/kg}$ as $\text{NaNO}_3$ )	Nitrite ( $\text{mg/kg}$ as $\text{NaNO}_2$ )
<i>Process samples taken during brewing</i>			
Water	ND <sup>b</sup>	45	0.4
Sweet wort	ND	60	0.7
Bitter wort	ND	115	0.1
Fermenting wort, 2 h	20	105	1.7
Fermenting wort, 12 h	140	85	6.7
Fermenting wort, 36 h	120	45	0.1
Finished beer (after racking, bottling and pasteurization)	100	40	ND
<i>Brewing ingredients</i>			
Malt (type a)	110	10	3.5
Malt (type b)	50	10	0.4
Hops (type a)	ND	360	2.1
Hops (type b)	450	1130	3.1
Hops (type c)	50	12 150	2.9
Yeast	400	15	1.4

supernatant (5 ml) was passed down three SAX columns prior to ATNC analysis of the eluent (1 ml). The solid material remaining after centrifugation was washed with further sulfamic acid (0.2 M, 3  $\times$  50 ml), and a portion (250 mg) of the nitrate-free solid analysed directly for ATNC.

Nitrate and nitrite in beers and worts and brewing water were determined by direct chemical reduction with chemiluminescence detection of nitric oxide (Cox, 1980; Walters *et al.*, 1986). Brewing ingredients (5 g) were extracted with potassium hydroxide (1.0 M, 50 ml), centrifuged and the supernatant analysed.

<sup>b</sup>ND, none detected subject to limits of 20  $\mu\text{g N-NO/kg}$  for ATNC and 0.1 mg/kg for sodium nitrite

<sup>a</sup>ATNC in beer were determined by direct chemical denitrosation and chemiluminescence detection of the released nitric oxide, using the group-selective procedure devised by Walters *et al.* (1978). To prevent the risks of artefact formation of ATNC and of false-positive response from nitrite and nitrate, beer samples (5 ml) were treated with sulfamic acid (0.2 M, 1 ml) for 5 min to destroy nitrite and passed down a strong anion exchange (SAX) column (Bond Elut, Analytichem) to remove nitrate. The eluent (1 ml) was added to refluxing ethyl acetate and treated sequentially with glacial acetic acid and 15% hydrogen bromide in acetic acid to determine ATNC, as described previously (Massey *et al.*, 1984b). This procedure was also used to analyse ATNC in brewing water. Wort samples (5 ml) were treated with sulfamic acid (0.2 M, 1 ml) and sulfuric acid (1.0 M, 0.2 ml) to counteract the malt protein buffer capacity and to ensure that the pH was below 2.0. Three SAX columns linked in series were used to prevent breakthrough of nitrate. Brewing ingredients (250 mg) with nitrate levels below 25 mg/kg were analysed directly for ATNC, as described previously (Massey *et al.*, 1984b). Ingredients (5 g) with higher nitrate levels, such as hops, were extracted with sulfamic acid (0.2 M, 100 ml) and centrifuged, and the super-

Detectable amounts of ATNC were also present in the malt, hops and yeast at concentrations up to 450  $\mu\text{g N-NO/kg}$ . However, in view of the dilution factor involved in brewing, the ATNC levels attributable solely to these ingredients would be below the 20  $\mu\text{g N-NO/kg}$  detection limit in the process samples. This is confirmed by the absence of ATNC in the sweet and bitter worts and the barely detectable concentration in the 2-h fermentation sample.

These results show that the levels of ATNC present in the finished beer derive from *N*-nitrosation reactions that take place in the fermenting wort and not from preformed compounds present in the brewing ingredients. This view is supported by the data in Table 1 for nitrite and nitrate, which show that nitrosating conditions exist in the fermenting wort. In fact, the nitrate concentration decreases markedly during fermentation, from 115 mg/kg to 45 mg/kg. This was accompanied by the formation of detectable, although transitory, amounts of nitrite. Comparison of the levels of nitrate and ATNC in the 2-h fermentation sample with those in the finished beer indicate that approximately 0.2% of the reduction in the level of nitrate was accounted for by formation of stable ATNC. The large majority of the nitrate was presumably consumed by reaction *via* nitrite with other components of the fermenting wort and also by loss to the headspace as nitrogen oxides.

Formation of ATNC during fermentation has also been observed during the production of other commercial beers at separate breweries in the UK (data not given). It seems probable that the nitrate reduction during fermentation is due to the normal adventitious presence in brewing yeasts of bacterial species with nitrate reductase activity. Bacterial nitrate reduction has been proposed by Weiner *et al.* (1975) and by Savel and Prokopova (1982) to account for the occurrence of nitrite observed in some brewing yeasts. The levels of bacteria required to produce ATNC are much less than those that would result in beer spoilage.

A number of procedures have proved successful in eliminating the formation of ATNC during fermentation. These include the use of freshly propagated yeast to avoid the build up of high levels of bacterial contamination that may occur in older yeast preparations. Acid washing of the yeast slurries prior to fermentation also inhibits ATNC formation, due to the acid-mediated inactivation of microbial species with nitrate reductase activity. Such treatment may affect the surface properties of the yeast, which can lead to a variety of difficulties such as incomplete fining (i.e., clarification). ATNC synthesis is also eliminated if nitrate-free brewing ingredients are employed. Details of these findings will be published elsewhere.

The biological significance of the presence of ATNC in beer cannot be evaluated until the identities of the putative *N*-nitroso compounds concerned are known. Proline is the single most abundant secondary nitrogen compound in beer, and *N*-nitrosoproline (NPRO) has been reported in concentrations of up to 23  $\mu\text{g/kg}$  (Massey *et al.*, 1982; Sen *et al.*, 1983). Recent findings in our laboratory (unpublished data) have shown that neither free nor peptide/protein-bound NPRO accounts for more than up to 10% of the ATNC. A number of *N*-nitroso compounds more polar than NPRO have, however, been observed chromatographically, and studies are currently in progress to characterize these species.

#### Acknowledgements

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# MAGNETIC SEMIPERMEABLE AQUEOUS POLYETHYLENEIMINE MICROCAPSULES FOR MONITORING *N*-NITROSATING SPECIES IN THE GASTROINTESTINAL TRACT

I.K. O'Neill, M. Castegnaro, I. Brouet & A.C. Povey

*International Agency for Research on Cancer  
150, cours Albert Thomas, Lyon, France*

Magnetic polyethyleneimine (PEI) microcapsules have been developed for trapping electrophilic intermediates in the gastrointestinal (GI) tract. The *N*-nitrosation of these microcapsules at acid pH was found to be linearly dependent on nitrite concentration, without a pH maximum and with efficient conversion to *N*-nitrosated products which were detected by a total *N*-nitroso assay procedure. The limit of detection is in the range 1-10 nmol *N*-nitroso compound, depending on microcapsule preparation conditions. Nuclear magnetic resonance (NMR) indicates that such *N*-nitrosation is favoured by incomplete protonation of the polyamine. Microcapsules administered orally to rats were recovered magnetically from faeces and showed extensive *N*-nitrosation when nitrite was administered in the drinking-water.

Magnetic semipermeable aqueous PEI microcapsules have been developed for trapping unstable electrophilic intermediates (Povey, 1985) as a means of investigating the GI tract, for three reasons: the incidence of GI-tract cancer is high, the GI tract is the site of greatest exposure to many carcinogens, and diet has a major influence on cancer in general. Subsequent to development of the *N*-nitrosoproline test (Ohshima & Bartsch, 1981), it appeared necessary to develop systems for monitoring endogenous *N*-nitrosation that would have high trapping efficiency, in competition with the GI-tract tissue and contents, and would allow direct investigation of colorectal nitrosation reactions, with the linearity of the trapping proportional to  $[\text{NO}_2^-]$  rather than to  $[\text{NO}_2^-]^2$ . The present investigation on the use of PEI microcapsules was aimed at such improvements in the monitoring of endogenous nitrosation.

## Development of semipermeable, magnetic, PEI microcapsules for in-vivo trapping of electrophiles

Apart from work by Sparks *et al.* (1971) on the microencapsulation of carbon for trapping uraemic metabolites, no technique has been available for trapping active species in the GI tract, protecting the product from further metabolism and permitting easy recovery. The method of Chang *et al.* (1966), in which cross-linked semipermeable polyamide membranes enclose aqueous interiors (cores), was adapted, by using excess PEI to function as a polymeric nucleophilic trap and magnetic fluids to allow easy recovery of the micro-

capsules from faeces. The resulting microcapsules have strong PEI-cross-linked poly(hexamethyleneterephthalamide) membranes, diameters in the range 15-50  $\mu\text{m}$  (Table 1), demonstrated stability within the GI tract and (so far) absence of toxic side-effects in experimental animals. PEI is highly soluble in aqueous solutions at all pH encountered *in vivo*, and, when given as millions of microcapsules by gavage to rodents, offers efficient scavenging possibilities. These microcapsules have been demonstrated to trap reactive products of *N*-methyl-*N*-nitrosourea, 1,2-dimethylhydrazine and benzo[*a*]pyrene in the rodent GI tract. Gradations of membrane properties and molecular weight of reactants have been studied to optimize the microcapsule trapping characteristics (Povey *et al.*, 1986 and unpublished results).

**Table 1. Physical properties of microcapsules and their reaction with nitrite<sup>a</sup>**

Type <sup>b</sup>	Core/mem- brane PEI ratio <sup>c</sup>	Mean diameter ( $\mu\text{m}$ ) <sup>d</sup>	Number of microcapsules used $\times 10^6$	Total N-NO measured in microcapsules (nmol NO)		
				Blank <sup>e</sup>	After 8 nmol nitrite	After $1.2 \times 10^4$ nmol nitrite
A	1.3	20	2.9	< 1	6.5	357
B	1.0	23	1.5	5	-	337
C	4.7	40	1.2	3.4	7.9	207
D	5.9	24	1.7	2.8	6.6	378

<sup>a</sup>Microcapsules containing 250  $\mu\text{g}$  releasable PEI treated with nitrite at pH 2.0, total volume 1.0 ml, shaking 2 h at 37°C; values not corrected for microcapsule blank

<sup>b</sup>Prepared with same amounts of PEI and terephthaloyl chloride in aqueous emulsion, but with different hexamethylenediamine concentrations in order to vary membrane characteristics, such as thickness and porosity

<sup>c</sup>Measured by ultrasonication of microcapsules followed by core PEI analysis by fluorescamine assay, and membrane PEI analysis by C/N elemental determination

<sup>d</sup>Measured on TA2 Coulter Counter

<sup>e</sup>Microcapsules treated in absence of nitrite

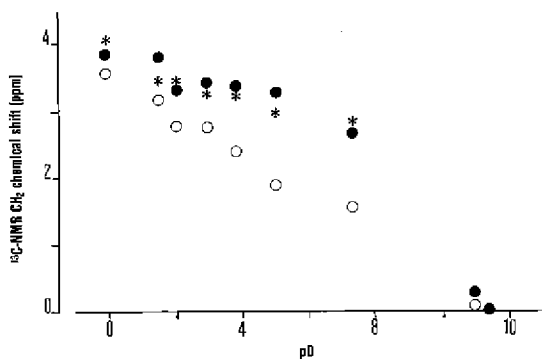
### *N*-Nitrosation of PEI

The PEI used presently has a molecular weight of 50 000, with a highly-branched tree-like molecular structure; the 1200 nitrogen atoms in each molecule are primary, secondary and tertiary amines in a 1:2:1 ratio (Horn, 1982). Since this large number of amines in a closely-spaced array might affect protonation and consequent *N*-nitrosation behaviour, the protonation of PEI was examined by <sup>13</sup>C-NMR spectroscopy, as conventional titration procedures have given conflicting results (Horn, 1982). Utilizing published assignments of the eight <sup>13</sup>C-NMR signals at pH 11 (Lukhovin *et al.*, 1973), the pH-dependent NMR titration of <sup>13</sup>C nuclei adjacent to primary, secondary and tertiary nitrogens (Fig. 1) showed that the secondary amines of PEI are incompletely protonated even at pH 1, due to prior protonation of tertiary nitrogens and the increasing difficulty to further protonate a molecule with >1000 closely adjacent positive charges. Using a limited amount of nitrite, it could be shown by <sup>13</sup>C-NMR spectroscopy that *N*-nitrosation occurs



predominantly at unhindered secondary amines (Fig. 2). The absence of prior reaction of primary amines is contrary to the expected nitrosation reaction, but was confirmed by efficient trapping of nitrite to yield *N*-nitrosated PEI (data given below).

**Fig. 1. pD-Dependent protonation of primary, secondary and tertiary amines of PEI as determined by  $^{13}\text{C}$ -NMR chemical shifts of adjacent methylene carbons at 88.2 MHz in  $\text{D}_2\text{O}$**

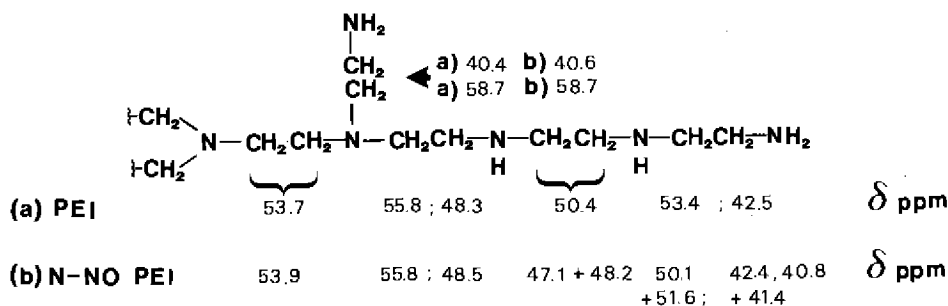


$^{13}\text{C}$ -NMR data for methylene carbon nuclei adjacent to primary amines (○) to secondary amines (\*), and to tertiary amines (●)

age; efforts are being made to minimize this in order to reduce the consequent effective detection limit of <1 to 10 nmol total *N*-nitroso compounds.

An improved method for determination of total *N*-nitroso compounds (Castegnaro *et al.*, 1986) was adapted to analysis of nitrosated PEI. Nitrosated products, treated for residual nitrite by a 1000-fold excess of sulfamic acid, added as small aliquots (up to 0.2ml) of aqueous PEI solutions or PEI microcapsule suspensions to 50 ml glacial acetic acid in the denitrosation procedure, gave no significant response in the Thermal Energy Analyzer (TEA) either in the cold state or on heating to reflux. On addition of hydrogen bromide/acetic acid there was a rapid TEA response, as anticipated for *N*-nitroso compounds. A microcapsule *N*-nitroso blank value of <1 to 5 nmol *N*-NO arises in part from the commercial PEI used in microcapsule preparation, but they are also probably formed during preparation and storage

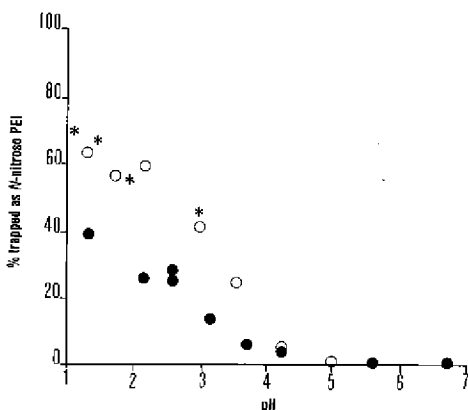
**Fig. 2. Representative formula for branched-chain PEI with 88.2 MHz  $^{13}\text{C}$ -NMR chemical shifts at pD 11.0 (a) before and (b) after *N*-nitrosation at pD 2.0**



### *N*-Nitrosation kinetics of PEI microcapsules

*N*-Nitrosation of PEI microcapsules was performed at a range of different pH in buffered solutions, shaking the suspensions at 37°C for 2 h to simulate probable microcapsule retention time in the stomach. The *N*-nitrosation increased from pH 5 to 1 with no apparent maximum (Fig. 3). The amount of nitrite converted to *N*-nitroso PEI (i.e., the *N*-nitrosation capacity) was limited by the number and type of the microcapsules used (Table 1). <sup>1</sup>H-NMR spectroscopic observation of the core PEI in intact nonmagnetic PEI microcapsules showed its chemical shifts to be changed by aqueous buffer in the same manner as unencapsulated PEI, i.e., the PEI in the microcapsule core PEI is buffered externally.

**Fig. 3. Fraction of nitrosating species trapped by microcapsules, type B, in relation to pH and nitrite concentration**



Type B microcapsules (Table 1, with 250  $\mu$ g releasable PEI equivalent and ultimate capacity of 337 nmol NO) incubated with either 48.5 (\*), 200 (O) or 485 (●) nmol nitrite in 1.0 ml solution

200  $\mu$ mol/l nitrite in the pH range for the normal healthy fasting stomach, there is less than a two-fold change in the incremental uptake of additional nitrite (Table 2), indicating no progressively large decrease in conversion of nitrite to analysable *N*-nitroso PEI. The apparent microencapsulated PEI capacity for *N*-nitrosation is less than one-third of the theoretical capacity of the incorporated PEI, calculated for unhindered PEI secondary amines, probably due to diminishing aqueous solubility of the exterior and more accessible branches on the PEI molecule as they become nitrosated. The NMR data show that some PEI are unprotonated at acid pH and thus analogous to weakly basic and rapidly *N*-nitrosated amines, but the *N*-nitrosation of PEI microcapsules is linearly dependent on nitrite concentration and, without a pH optimum, follows the behaviour of amides (Mirvish, 1975). Since proteins may be an important substrate for endogenous *N*-nitrosation, and an ideal biological monitoring system should yield data *directly* related to the concentration of species being monitored, PEI microcapsules would appear to be chemically appropriate for in-vivo indication of endogenous *N*-nitrosating species.

Microcapsules containing a total of 250  $\mu$ g releasable PEI ( $1.5 \times 10^6$  type B or  $1.2 \times 10^6$  type C microcapsules, Table 1) were nitrosated at pH 2 with nitrite concentrations ranging from 5 to 12 000  $\mu$ mol/l. The extent of nitrosation found in the microcapsules represented up to 90% of nitrite added and increased linearly over two orders of magnitude of the concentration of nitrite before reaching plateau values set by the microcapsule capacity for *N*-nitrosation (Fig. 4). When the nitrosation was performed with a fixed amount of nitrite using decreasing numbers of one type of microcapsule, the *N*-nitrosation capacity strictly controlled the fraction of nitrite converted into *N*-nitroso PEI.

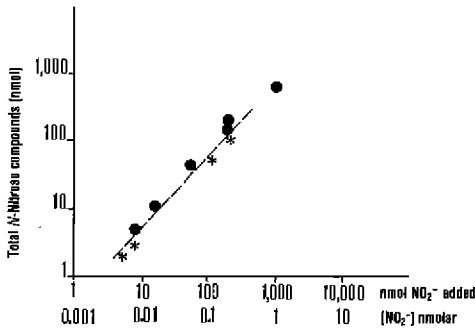
The trapping efficiency at very low concentration of nitrite (Table 1) is obscured by the significant blank values but seems unrelated to microcapsule *N*-nitrosation capacity and may be controlled by membrane parameters now being investigated. Below the nitrosation capacity and at up to

**Table 2. Incremental percentage of nitrosating agent trapped by microcapsules in relation to pH and quantity of nitrite<sup>a</sup>**

pH	0 - 49 nmol nitrite	49 - 200 nmol nitrite	200 - 485 nmol nitrite
1.3 + 0.2	71%	67%	20%
2.1 + 0.1	56%	60%	1%
3.1 + 0.1	45%	40%	1%

<sup>a</sup>See legend to Figure 3 for conditions.

**Fig. 4. Extent of trapping by type B (●) and type C (\*) microcapsules (see Table 1) of nitrosating species at pH 2.0**



Quantity of total *N*-nitroso compounds found as a function of nitrite concentration by 250  $\mu$ g releasable PEI equivalent. Horizontal broken lines indicate apparent *N*-nitrosation maxima for the two microcapsule types.

analysis for total *N*-nitroso compounds and iron content was performed after magnetic extraction and washing of microcapsules. Substantial *in-vivo* trapping of nitrosating species was found (Table 3), with large interindividual differences. The total amounts of *N*-nitroso compounds, in all but one case, were far greater than the 0.1-0.3 nmol *N*-nitroso compounds found in microcapsules excreted by animals that had not received nitrite. The microcapsule blank value may contribute substantially, thus setting a detection limit.

It is evident that free access to nitrite in drinking-water resulted in uncontrolled exposure to nitrosating agent, and some animals that had 15-h prior exposure excreted micro-

#### **In-vivo trapping of nitrosating species**

Previous experience with these types of microcapsule (Povey, 1985) has shown that up to 60% are recovered by a magnetic tool from mixed aqueous suspensions with rat faeces and food. Male BDIV rats were housed individually in metabolic cages, fasted overnight prior to gavage with an aqueous microcapsule suspension *via* a stomach tube, and then immediately provided with biscuit food. It is believed that microcapsules pass from the stomach about 2 h after gavage; thus, access to nitrite-containing drinking-water was given either 15 h before gavage, at gavage, or 6 h later. Faeces were collected into acidified ammonium sulfamate for 24 h, and

**Table 3. Dependence of microcapsule trapping of nitrosating species on time of nitrite administration to BDIV rats and number of microcapsules given in first 24 h after microcapsule gavage**

Number of microcapsules <sup>a</sup> × 10 <sup>6</sup>	Access <sup>b</sup> to nitrite commenced (h)	Total <i>N</i> -nitroso found in excreted microcapsules <sup>c</sup> (nmol)
2	-15	44, 18
2	0	29, 0.7
2	+6	0, 6, 17, 52
6	-15	109, 57
6	0	60, 20

<sup>a</sup>Type C microcapsules (see Table 1); 2 × 10<sup>6</sup> microcapsules equivalent to 250 µg releasable PEI

<sup>b</sup>*Ad libitum* access to drinking-water; changed to 1000 ppm sodium nitrite in drinking-water either 15 h before, during or 6 h after gavage

<sup>c</sup>Blank values were 0.1-0.3 nmol total *N*-nitroso compounds for animals receiving 2 × 10<sup>6</sup> microcapsules and no nitrite.

genous nitrosation, and to reduce the large interindividual variations that arise not from the novel nature of the PEI trapping process but from their localized dispersion in GI-tract contents. It is presently unclear whether *N*-nitrosation of microcapsules given 6 h before access to nitrite indicates *N*-nitrosation in the lower GI tract or incomplete clearance from the stomach.

#### Acknowledgements

We gratefully acknowledge Mrs M. Wisez for secretarial help with this manuscript, Mr J.-C. Béréziat for assistance with animal treatment, and the National Institutes of Health for grant 1 ROI CA39417-01 in support of this work.

capsules that were completely nitrosated. Iron analysis of recovered microcapsules showed recoveries in the range 5-20% of those administered; parallel work has recently shown that faeces should be collected for 48 h. A suggestion of dependence on microcapsule quantity was found in these preliminary experiments, with a probable limiting effect of microcapsule capacity. Parallel work on in-vivo trapping of other electrophilic species (Povey *et al.*, 1986), including *N*-methyl-*N*-nitroso-urea (unpublished data), has demonstrated a dependence of microcapsule trapping on time, dose and GI-tract site. Work is in progress to compare this system with other indicators of endo-

# LASER PHOTOFRAGMENT SPECTROSCOPY: A NEW TECHNIQUE FOR THE DETECTION OF N-NITROSAMINES AND OTHER NITROSO COMPOUNDS

M.R.S. McCoustra & J. Pfab

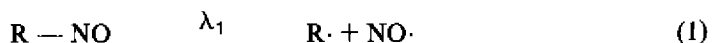
*Department of Chemistry, Heriot-Watt University, Riccarton,  
Edinburgh, EH14 4AS, Scotland, UK*

The potential of laser photofragment spectroscopy (LPS) for the detection and discrimination of nitrosamines, alkyl nitrites, thionitrites and C-nitroso compounds has been evaluated. The technique combines high sensitivity with the dramatic specificity of high-resolution spectroscopy and utilizes pulsed laser photodissociation with synchronized detection of the NO photofragment by laser-induced fluorescence (LIF). Using supersonic molecular beam sampling and wavelength tunable photodissociation, LPS can generate well-resolved finger-print spectra characteristic of the parent nitroso compound

Most N-nitrosamines can be determined reliably at very low levels by gas or liquid chromatography in conjunction with a thermal energy analyser (TEA) (Fine & Rounbehler, 1975). Apart from mass spectrometry, there is no spectroscopic technique available for the characterization of unknown nitrosamines that combines specificity and high sensitivity. We have developed a new laser technique for the detection of NO-containing molecules that offers the dramatic specificity of a high-resolution spectroscopic technique and good sensitivity.

## Principle of LPS detection

It is well known that nitroso compounds absorb light in the 220-700 nm region and readily photodissociate in the gas phase to radicals and NO in its electronic ground state ( ${}^2\pi_i$ ). With focused pulsed laser beams, the absorption and photofragmentation steps can be made highly efficient. The NO photofragment is detected by LIF using a dye laser of narrow band width. LIF is a well-developed high-resolution spectroscopic technique that has been applied to many other di- and tri-atomic gas phase species, for example, OH and CN. The probe laser pumps the diatom to a fluorescent upper electronic state, and the resonance fluorescence from the pumped level is detected by a photomultiplier and provides the signal. The sequence of events involved in the LPS detection scheme can be summarized as follows:



The NO-containing species is photolysed by a pulsed laser at wavelength  $\lambda_1$ , chosen to coincide with an electronic absorption of the molecular parent. The delayed synchronized-

pulsed probe laser is tuned to a molecular resonance of NO ( $\lambda_2$ ) produced by the preceding dissociation pulse. This technique has been used previously to study the dynamics of the photodissociation of NO-containing molecules (Roellig *et al.*, 1980; Zacharias *et al.*, 1980; Pfab *et al.*, 1983). The A state of NO used in the LIF probe scheme gives rise to the well known  $\gamma$  emission bands between 220 and 300 nm. This state is accessed in our work by a two-photon process in the 450-490-nm region. Detection of NO by two-photon LIF has relatively low sensitivity ( $10^{10}$ - $10^{11}$  molecules per  $\text{cm}^{-3}$ ) due to the small two-photon absorptivity of NO. Single-photon LIF detection of NO *via* the A state, in contrast, is capable of  $10^4$  to  $10^5$  times higher sensitivity but requires equipment for the generation of tunable laser radiation near 225 nm.

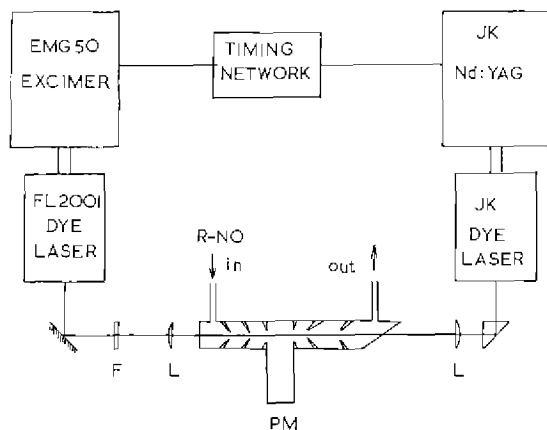
### Apparatus and sampling methods

Two types of experimental set-up were used. A simplified lay-out, showing experiments conducted with room-temperature sampling of the NO-containing molecules is presented in Figure 1. Pressures of 1 to 200 mTorr of the nitroso compound were admitted to the fluorescence cell, maintaining a gentle flow of the vapour to avoid contamination by photolysis products and to ensure a steady supply of pure vapour. A pulsed Nd-YAG pumped dye laser (JK 2000) provided tunable laser light between 550 and 750 nm for photolysis of C-nitroso compounds. Alternatively, the fixed frequency tripled output of the YAG laser was used for photolysis of the alkyl nitrites and nitrosamines at 355 nm. *N*-Nitrosomethylecyanamide photolysis at 308 nm was accomplished with a XeCl excimer laser (Lambda Physik EMG 50E), which also pumped the probe laser for the two-photon LIF detection of NO near 450 or 470 nm (Lambda Physik, FL2002). A delay generator linked to a crystal-controlled oscillator provided synchronization of the dissociation and probe laser pulses in the nanosecond regime. The dissociation and probe beams were aligned co-linearly and focused to the same spot in the centre of the cell. Nitrogen dioxide and the alkyl thionitrites photodissociate effectively in the 450 to 490 nm range, and no separate dissociation beam was required here. The ultraviolet fluorescence emitted from the probe volume was collected, filtered and imaged on a solar blind photomultiplier. The photomultiplier signal was processed using conventional gated signal acquisition and averaging techniques.

The second set-up used for LPS detection of nozzle-cooled molecules will be described in detail elsewhere. Briefly, the gaseous nitroso compound is mixed with excess argon or helium gas and expanded through a 0.5-mm orifice into a vacuum chamber. Supersonic expansion by means of a solenoid valve produces a pulsed molecular beam of the 10-20K cold nitroso compound. The molecular beam is crossed ten nozzle diameters downstream of the orifice by the co-linearly aligned and focused dissociation and probe beams. Two-photon LIF of the NO photofragment is detected at right angles, as before.

With either sampling technique, the probe laser generates two-photon LIF spectra of the nascent NO fragment on tuning it through the  $\gamma(0,0)$  band at 450 nm or the  $\gamma(0,1)$  band at 470 nm. Figure 2 presents an example, showing a small part of the high-resolution two-photon LIF spectrum of NO from the 355 nm photolysis of *N*-nitrosodimethylamine in a supersonic cold beam of argon. The intensity of each rovibronic transition in these LPS spectra is proportional to the number density in molecules per  $\text{cm}^3$  of the probed rotation-vibration quantum state of NO. Thus, the signal observed for one particular transition depends on the amount of NO produced by photolysis and on the way the quantum states

**Fig. 1. Simplified scheme of experimental arrangement used for pulsed LPS of gaseous nitroso compounds at room temperature; PM, photomultiplier**

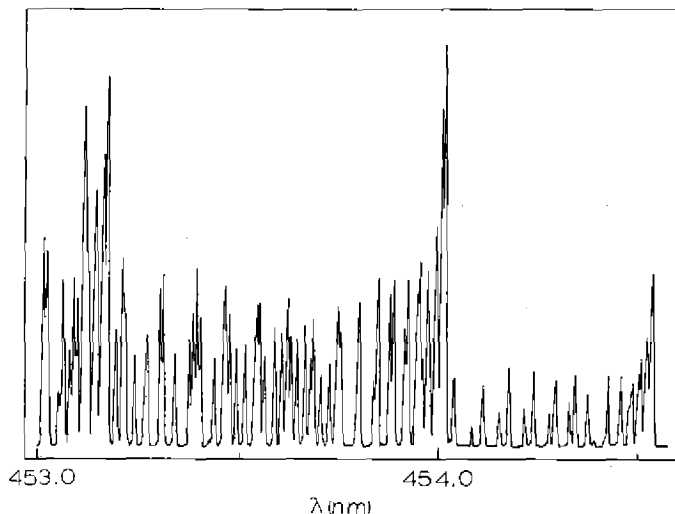


for the vibration and rotation of the nascent NO are populated. These quantum state distributions are controlled by the dynamics of the photodissociation process and are not affected much by collisional processes.

#### Detection limits and specificity

The lower detection limits were evaluated from signal-to-noise ratios observed in the NO LPS spectra and the pressure of the parent nitroso compound (Table I). In several cases, the absorption and dissociation steps could be optically fully saturated, such that each NO-containing molecule in the probe volume produced one molecule of NO. The detection limit of LPS is clearly dominated by the sensitivity of the probe technique.

**Fig. 2. Two-photon LIF spectrum of NO ( $v = 0$ ) from the 355 nm photodissociation of *N*-nitrosodimethylamine in a nozzle-cooled supersonic molecular beam of argon**



With the two-photon LIF technique used, sensitivity is limited to  $1 \times 10^{-13}$  mol/cm<sup>3</sup> for NO. The main factors that reduce the sensitivity of the LPS detection scheme are lack of absorption of the parent and broad vibrational and rotational population distributions of the nascent photofragment. In the nozzle-cooled beam, the  $n, \pi^*$  absorption spectra of

**Table 1.** Nitroso compounds examined by LPS and extrapolated detection limits

Nitroso compound <sup>a</sup>	$\lambda_1$ (nm) <sup>b</sup>	$\lambda_2$ (nm) <sup>b</sup>	Condition <sup>c</sup>	Detection limit (mol/cm <sup>3</sup> )
NDMA	355	450	MB-Ar,He	$2 \times 10^{-12}$
NMCA	355	450	MB-Ar,He	$1 \times 10^{-11}$
NMCA	308	380 <sup>d</sup>	RT	$1 \times 10^{-15}$
CH <sub>3</sub> SNO	450	450	RT	$8 \times 10^{-13}$
<i>t</i> -BuSNO	450	450	RT	$3 \times 10^{-13}$
CH <sub>3</sub> ONO	355	470	MB-Ar	$3 \times 10^{-11}$
<i>t</i> -BuONO	355	470	MB-Ar	$9 \times 10^{-12}$
CClF <sub>2</sub> NO	550-750	450	MB-Ar	$6 \times 10^{-12}$
CNP	550-750	450	RT	$2 \times 10^{-12}$
NO <sub>2</sub>	450	450	RT	$5 \times 10^{-13}$
NO <sub>2</sub>	450	450	MB-Ar	$9 \times 10^{-12}$

<sup>a</sup>NDMA, *N*-nitrosodimethylamine; NMCA, *N*-nitrosomethylcyanamide; CNP, 2-chloro-2-nitrosopropane

<sup>b</sup> $\lambda_1$ : dissociation wavelength;  $\lambda_2$ : LIF probe wavelength

<sup>c</sup>Sampling conditions: MB-Ar,He, nozzle-cooled molecular beam with Ar or He as expansion gas; RT, neat vapour at room temperature

<sup>d</sup>Detection of CN rather than NO fragment by single-photon LIF from the B state of CN

nitroso compounds are highly structured, presenting narrow spectral features that constitute characteristic finger-prints specific to individual compounds. Optimal sensitivity requires a tunable dissociation laser the wavelength of which can be tuned into coincidence with a strong absorption feature of the nitroso compound.

Control of the wavelength of the dissociation laser makes the LPS technique much more selective than the TEA procedure. Clearly, the selectivity is highest when the sample is cooled by supersonic nozzle expansion and when the dissociation wavelength is tunable. *N*-, *S*-, *O*- and *C*-nitroso compounds can be discriminated also by differences in their NO spectra. Thus, NO from *C*-nitroso compounds is vibrationally and rotationally cold, while NO from the alkyl nitrites is exceedingly hot.

### Potential future applications

Our results indicate that the sensitivity of LPS can be improved to  $10^6$  molecules per cm<sup>3</sup> and that highly resolved finger-print spectra of the parent nitroso compound can be recorded in supersonic nozzle-cooled beams. While the technique is some hundred to thousand times less sensitive than mass spectrometry, it is more selective than TEA detection, does not require ultra-high vacuum and shows promise for the characterization of volatile and nonvolatile nitroso compounds by optical high-resolution spectroscopy.

### Acknowledgements

We thank the Carnegie Trust of the Universities of Scotland for a scholarship (to M.R.S. McCoustra) and the UK Science and Engineering Research Council for a grant.



# IN-VITRO ASSAYS TO DETECT ALKYLATING AND MUTAGENIC ACTIVITIES OF DIETARY COMPONENTS NITROSATED *IN SITU*

S.E. Shephard, M.E. Hegi & W.K. Lutz

*Institute of Toxicology, ETH and University of Zurich,  
CH - 8603 Schwerzenbach, Switzerland*

Nitrosation of dietary components has been combined with the 4-(*para*-nitrobenzyl)pyridine (NBP) colorimetric test for screening alkylating agents and with the Ames test for the detection of mutagenic activity. This allowed the investigation of short-lived nitrosation products of dietary components which generate electrophilic degradation products requiring no metabolic activation (natural amino acids and some derivatives, ureas, guanidines, primary alkyl and aryl amines). In a first system, precursor, nitrous acid and NBP were present simultaneously. All amino acids tested, except glutamic acid and glutamine, gave positive results. The reactivities spanned more than three orders of magnitude, with the aromatic amino acids and methionine the most active; two primary amines, tryptamine and histamine, were also strongly reactive. All guanidines tested, except the amino acid arginine, gave negative results. A second system consisted of two phases: NBP was added only after destruction of residual nitrite and adjustment of the pH to neutrality. This system was useful for the study of ureas, which are stable in acid but not in neutral media. The range of responses covered more than two orders of magnitude. Most amino acids and primary amines also gave positive results, but could be assessed only after analysing the kinetics of the competing reactions and choosing appropriate reaction times. In a third system, *Salmonella typhimurium* strain TA100 replaced NBP. Representatives of the class of amino acids, ureas, the primary amine tryptamine, and aniline became highly mutagenic upon nitrosation. Methylguanidine was only weakly mutagenic under the present assay conditions. The results indicate that further studies with unstable nitrosation products of dietary components are required to understand more thoroughly the role of endogenous nitrosation in gastric cancer.

In studies of compounds that could produce genotoxic carcinogens upon nitrosation, the precursors that lead to unstable *N*-nitroso compounds have been given relatively little attention. One reason for the lack of interest has been the problem of handling reactive compounds. A recent report on studies of precursors in the human diet pointed out the need for additional effort on ureas and amides, aryl amines, guanidines and amino acids (Shephard *et al.*, 1987 and this volume). Test systems have thus been developed in which labile nitrosation products formed in nitrosation assays can be analysed for alkylating or mutagenic activity *in situ* or immediately after their generation.

NBP was introduced by Epstein *et al.* (1955) as an analytical reagent for alkylating agents. Alkylation of the pyridine nitrogen followed by alkaline deprotonation of a benzylic hydrogen results in a dark blue-violet chromophore with an absorption maximum at 580 nm. The applications of this reagent to detect activation-independent carcinogens,

including *N*-nitroso compounds, have been compiled by Archer and Eng (1981) in the introduction to a paper describing the formation of alkylating intermediates from *N*-nitrosodiethylamine by a chemical oxidation system. The modifications described below include a system in which precursors are nitrosated in the presence of NBP in order also to trap highly labile alkylating reaction products.

*Salmonella* bacteria have for some time been used as indicator organisms to detect activation-independent mutagenic reaction products formed in a nitrosation assay, without extracting *N*-nitroso compounds from the incubation mixture. Positive results with defined precursors have been reported with guanidines (Endo *et al.*, 1974), aminoantipyrine and aniline (Boido *et al.*, 1980), ureas and carbamates (Takeda & Kanaya, 1982), tryptophan, tryptamine and 5-hydroxytryptamine (Gatehouse & Wedd, 1983), ranitidine (De Flora *et al.*, 1983) and diverse amine drugs (Andrews *et al.*, 1984), as well as with indole-3-acetonitrile from Chinese cabbage (Wakabayashi *et al.*, 1985a). We describe a modification of the system which allows bacteria to be present when the nitrosation reaction mixture is neutralized.

#### **Alkylation of NBP *in situ*: NBP one-phase system**

In our new system, the nitrosation and alkylation with NBP are allowed to proceed simultaneously. In order that all components can be brought into solution, the solvent system has to be a compromise between an acidic buffer (250 mM phosphate, pH 2.5) and an aprotic solvent mixture (ethylene glycol plus acetone). Under standard conditions at 37°C, a concentration of 40 mM is used both for the test compound and for nitrite; NBP is added at 47 mM. At intervals of 15 min to 1 h, aliquots of the reaction mixtures are made basic by the addition of triethylamine with vigorous mixing. Absorption at 580 nm is measured after exactly 30 and 60 sec, and the absorbance at  $t = 0$  sec is calculated by extrapolation, assuming a first-order decay of the colour due to instability of the NBP-test compound adduct.

This one-phase system seems to be highly versatile, because it makes possible the instantaneous trapping of electrophiles formed with nitrous acid. Results are shown in Table 1. Rates of reaction ( $A_{580}/\text{min}$ ) were expressed in relation to glycine (set at 100%). All  $\alpha$ -amino acids except glutamic acid and glutamine produced a positive result. The reactivities spanned more than three orders of magnitude, with the aromatic amino acids and methionine the most active. Acetylation of the amino group resulted in complete loss of alkylating activity; modification of the carboxylic acid function, however, gave rise to an increased response, indicated by the reaction rates of an ester and of a dipeptide of glycine. The dipeptide sweetening agent, aspartame (aspartylphenylalanine methyl ester), was the most potent alkylating agent under the present assay conditions. Ureas were not investigated in this system because *N*-methyl-*N*-nitrosourea was too stable in acid to react with NBP. All guanidines tested also gave negative results. Some primary amines containing an aromatic moiety were more effective alkylating agents than glycine, whereas methylamine ranked at 40% and propylamine reactivity was below the limit of detection. Data obtained with the aryl amine aniline are as yet inconclusive because the red colour observed must first be assigned to either side reactions or formation of other NBP-adduct chromophores.

#### **NBP two-phase system**

In the stomach, nitroso compounds formed under the acidic conditions of the lumen can diffuse into the lining cells, where they encounter neutral pH. In order to simulate this type

**Table 1. Alkylating potential (measured by the NBP reaction) and mutagenicity in the Ames test of dietary components incubated with nitrous acid**

Compound	NBP one-phase rate of reaction (% glycine)	NBP two-phase overall reactivity (% methylurea)	Ames test, two-phases (revertants/ $\mu$ mol) <sup>a</sup>
<b>Amino acids</b>			
Tryptophan	2300	ND <sup>b</sup>	2000 <sup>c</sup>
Methionine	1100	20	90
Tyrosine	400	ND	450 <sup>c</sup>
Glycine	100	16	260
Arginine	54	<2	24
Lysine	30	ND	<1
Glutamic acid	<1	ND	<1
<b>Amino acid derivatives and dipeptides</b>			
<i>N</i> -Acetylglycine	<0.1	<1	60 <sup>d</sup>
Glycine ethyl ester	150	10 000	2000 <sup>c</sup>
Glycylglycine	300	500	ND
Aspartame	8000	4200	200
<b>Ureas</b>			
Methyl urea	<0.1	100	170 <sup>c,d</sup>
Carbamoyl $\beta$ -alanine	ND	120	5900
Citrulline	7 <sup>e</sup>	6	20 <sup>f</sup>
Methylhydantoin	ND	<0.3	ND
<b>Guanidines</b>			
Methylguanidine	<0.1	<2	23 <sup>d</sup>
Creatin(in)e	<0.1	<2	<1
<b>Primary amines</b>			
Tryptamine	900	8	(pH 3.4) 7800
Histamine	450	pink colour	18
Methylamine	40	ND	<1
Propylamine	<1	ND	ND
<b>Aryl amine</b>			
Aniline	red colour	red colour	(pH 3.4) 2400 <sup>c,d</sup>

<sup>a</sup>Based on amount of precursor incubated<sup>b</sup>ND, no data<sup>c</sup>Derived from 0.08 or 0.8 mM initial concentration; high toxicity to bacteria at higher levels<sup>d</sup>Overproportional mutagenic activity at higher concentrations tested<sup>e</sup>Positive response probably due to amino acid function<sup>f</sup>pH 3.4

of two-stage process, a protocol involving two phases was worked out. First, test compound and nitrite were incubated at pH 2.5 for up to 60 min. Residual nitrite was destroyed by addition of amidosulfonic acid for 5 min, and NBP was added in a slightly alkaline amphiphilic solvent mixture to raise the pH to neutrality. Aliquots were taken at various times up to 90 min, triethylamine was added and the absorbance at 580 nm was measured immediately.

In this system, the overall response is dependent on a number of reaction rates: that of the formation and decay of *N*-nitroso compounds in acid, that of the *N*-nitroso compounds with NBP and other nucleophiles under neutral conditions, and the stability of the NBP adduct in neutral solution. The colour observed is therefore highly dependent on the reaction periods chosen. This complicates the experiment, but it allows analysis of reaction kinetics, so that the experimental findings can be extrapolated to the situation in humans.

The results derived from a 10-min/10-min incubation are shown in Table 1. Methylurea was used as the standard precursor and set at 100 percent, because the nitrosoureas are stable in acid and develop an alkylating potential only in neutral or alkaline solution. The optimal conditions for amino acids and primary amines have not yet been established. It is noteworthy, however, that the amino acid derivative *N*-acetylurea gave negative results at a limit of detection of 1% methylurea, whereas the formation of esters or peptides resulted in a markedly increased response. As with the one-phase system, all guanidines gave negative results.

#### Nitrosation assay procedure followed immediately by the Ames test

The alkylating potential does not indicate whether a reactive product can also penetrate biological membranes and react with DNA or whether the resulting adduct is biologically relevant. In order to investigate these questions, *S. typhimurium* TA100 was used as an indicator organism for genotoxicity. Because low pH is toxic to the bacteria and because nitrite is mutagenic, the nitrosation reaction could not be performed in the presence of the bacteria. A one-phase system was therefore impossible, but the conditions used in the two-phase system could be adapted.

In our improved protocol, test compound (normally at 8 and 80 mM) was incubated with nitrite (80 mM) for 30 min at 37°C in a 20 mM phosphate buffer pH 2.4 (amino acids and derivatives, ureas and guanidines) or pH 3.4 (primary amines, aniline, and citrulline). Residual nitrite was destroyed with amidosulfonic acid (1.1 molar equivalents; 5 min, including a sterile filtration). The pH was brought to near neutrality by adding the reaction mixture in 300 mM phosphate buffer pH 8, to which the bacteria had been added 1 min before. This procedure ensured the presence of the bacteria as soon as the pH was neutral. Incubation with the bacteria was continued for 30 min to increase the sensitivity of the Ames test. Then, the bacteria were plated to score for revertants, and an aliquot was assayed for survival. This control was essential for correct interpretation of the results, because it was found that strong mutagenicity was almost always paralleled by potent cytotoxicity, so that only data in which survival was good could be evaluated quantitatively. As a control for mutagenicity of the test compound without nitrosation, each test compound was also incubated with nitrate instead of nitrite; negative results were obtained in all cases.

Some results are shown in Table 1. For a quantitative comparison with the mutagenic potency of other chemicals, the data are expressed as induced revertants per  $\mu\text{mol}$  precursor. Since, for instance,  $\beta$ -propiolactone gives rise to 4000 revertants per  $\mu\text{mol}$ , the mutagenic potency of many dietary precursors incubated with nitrite is quite remarkable.

With increasing concentrations of the precursors marked with footnote<sup>d</sup> in Table 1, an overproportional increase in the mutagenic response was found. Such nonlinearity was never observed with NBP alkylation; thus, it is probably related to the specific indicator system, i.e., the use of bacteria. It remains to be elucidated whether processes other than alkylation are responsible for this additional mutagenic effect. If similar reactions are possible in stomach lining cells, this would add a new dimension to nitrite risk evaluation.

### Conclusions

The data presented clearly show that a great number of dietary components acquire electrophilic and mutagenic potential upon incubation with nitrous acid. For many classes of precursors, the response was much higher than anticipated.

The NBP one-phase system makes possible an overview of the various dietary precursor classes. Such information might facilitate the choice of compounds for supplementary *in-vivo* tests. The tests could include investigations of DNA binding in the stomach after treatment of animals with precursor and nitrite. Preliminary attempts have been made in this laboratory with a primary amine, methylamine, and with the urea compound dicarbamoylputrescine. Oral administration of <sup>14</sup>C-methylamine to rats, followed immediately by gavage with nitrite, resulted in the formation of 7-methylguanine in DNA isolated from the stomach (Huber & Lutz, 1984).

The two-phase systems are necessary for the biological interpretation of the data because they allow analysis of the kinetics of the various reactions. Such information is basic to the understanding of the underlying chemical processes and a prerequisite for extrapolating from the conditions used *in vitro* to the situation prevailing in the human stomach *in vivo*.

## **BIOLOGICAL EFFECTS**

# NITROSAMINE-INDUCED CARCINOGENESIS: EXPERIMENTAL MODELS FOR HUMAN MALIGNANCIES OF MULTIFACTORIAL ORIGIN

D. Schmähl

*Institute of Toxicology and Chemotherapy, German Cancer Research Center,  
Im Neuenheimer Feld 280, D-6900 Heidelberg, Federal Republic of Germany*

Carcinogenesis induced in humans by chemical agents is usually a process in which several compounds are involved, which exert their action on the target organ either concomitantly or sequentially. Exceptions are comparatively rare occupational cancers, in which exposure to only one chemical agent was found to be responsible, such as  $\beta$ -naphthylamine in urinary bladder carcinogenesis or vinyl chloride in the induction of haemangiosarcomas. In drug-induced carcinogenesis — for instance, by alkylating cytotoxic agents — there are also case reports in which a single agent induced so-called second tumours (Schmähl *et al.*, 1982). However, most other chemically-induced malignancies in humans, such as bronchial carcinoma, are due to a number of agents. This malignancy, which is mostly caused by inhalation of tobacco smoke, cannot be attributed to one agent, because tobacco smoke contains many carcinogenic compounds, each of which is to a certain degree involved in carcinoma formation. Our own investigations and assessment indicate that carcinogenic polycyclic aromatic hydrocarbons (PAH) in tobacco-smoke condensate play a decisive role in bronchial carcinogenesis. The PAH are present in tobacco smoke not as single agents but as a mixture; their effect is thus a combination effect. Further clear examples of combination effects occur in drug-induced carcinogenesis; for instance, chronic application of tar ointments and X-rays in the treatment of advanced eczemas led to the induction of skin cancer. Combined administration of different alkylating anticancer agents is also considered to result in combination effects in carcinogenesis. Finally, there are combined actions of carcinogenic viruses and carcinogenic chemicals. Combination effects are particularly relevant in occupational medicine, for instance, the combination of asbestos and tobacco smoke, and in environmental carcinogenesis.

When designing experiments to investigate combination effects in carcinogenesis, a number of factors has to be taken into account. When two or more agents are tested for their pharmacodynamics, many interactions are possible: the components may influence not only each other in their action on the target organ, but they may also modify the pharmacokinetics in a relatively nonspecific manner, which may in turn affect the target organ.

Modification of pharmacokinetics can also be induced by such nonspecific factors as diet, ambient temperature and day-night rhythm. One example is minimization of the carcinogenic effects of 4-dimethylaminoazobenzene by reductive cleavage of the azo bridge, which can be influenced by the dietary content of lactoflavin (Consbruch & Schmähl, 1952).

A second example is the inhibition by ascorbic acid of intragastric formation of nitrosamines (Ivankovic *et al.*, 1974). Such phenomena, however, are not considered strictly to be combination effects. The effects of several agents on a target organ can be promoted or inhibited in an additive or overadditive way, but they may also be exerted independently.

In order to investigate and compare the effects of several agents in combination, the biological activity of each must be determined prior to the combination experiment. No strictly quantitative experiment on combination effects has, to my knowledge, yet been carried out. This paper reports on our experiments on combination effects in chemical carcinogenesis. We have published more than 30 papers on this subject, in which fundamental basic toxicological research has played the main role. The following questions were dealt with in particular:

(1) Does concomitant administration of carcinogenic agents with identical organotropism result in addition of effects (Schmähl & Thomas, 1962, 1965; Schmähl, 1970; Schmidt *et al.*, 1976; Schmähl *et al.*, 1977)? Recent experiments (Berger & Schmähl, this volume) were designed to answer the question of whether, under strictly quantitative conditions, minimum doses also add up when hepatotropic carcinogens of different chemical structure are administered concomitantly.

(2) Is there addition of carcinogenic effects when carcinogens of different organotropism and cytotropism are administered concomitantly (Schmähl & Thomas, 1963, 1965; Schmähl *et al.*, 1964)?

(3) Can the carcinogenic effect of known carcinogens be enhanced or inhibited by noncarcinogenic agents that influence the function and structure of the target organ, when administered concomitantly (Schmähl *et al.*, 1965; Ivankovic *et al.*, 1972; Schmähl & Krüger, 1972; Schmähl *et al.*, 1974; Habs & Schmähl, 1976; Schmähl, 1976; Schmähl *et al.*, 1976a,b; Habs *et al.*, 1980; Habs & Schmähl, 1981, 1984; Narisawa *et al.*, 1984)?

(4) Can the carcinogenic effect be modulated by modification of endogenous functions, such as immunosuppression, immunostimulation or corticosteroid substitution (Schmähl *et al.*, 1971, 1974; Habs & Schmähl, 1976; Schmähl *et al.*, 1976a,c; Habs *et al.*, 1981; Wagner *et al.*, 1982)?

(5) Can carcinogenic effects be inhibited by specific or nonspecific 'anticarcinogenic' agents? Experiments include inhibition of cyclophosphamide-induced urinary bladder carcinogenesis by administration of mesna (Habs & Schmähl, 1983; Tacchi *et al.*, 1984) and inhibition of the carcinogenic effects of certain compounds by administration of retinoids (Schmähl & Habs, 1978, 1981).

The following parameters can be used to assess additive syncarcinogenic effects:

- (a) tumour incidence;
- (b) reduction of induction time in combination experiments;
- (c) doses needed to induce tumours;
- (d) change of organotropism;
- (e) histology of tumours induced; and
- (f) biological behaviour of tumours, e.g., metastasizing tendency.

The same parameters can also serve to assess inhibition of carcinogenesis.

Our experimental results are summarized in Tables 1-5.



**Table 1. Carcinogenesis by single or concomitant administration of two hepatotropic carcinogenic agents**

Treatment <sup>a</sup>	Daily dose (mg/kg)	No. of animals	Animals with tumours		Total dose (mg/kg)	Induction time (days)
			No.	%		
NDEA	3	28	28	100	700	233
DAB	33	23	18	78	7770	235
NDEA + DAB	3	38	36	95	460	153
	33				5160	
NDEA + liver damage	3	19	14	76	755	252

<sup>a</sup>NDEA, *N*-nitrosodiethylamine; DAB, 4-dimethylaminoazobenzene

**Table 2. Occurrence of tumours after chronic epicutaneous administration of different PAH combinations in mice (from Schmidt *et al.*, 1976)**

Treatment	Single dose (µg)	No. of animals	Mean life expectancy (weeks)	Histological diagnosis at site of application			Animals with tumours (%)
				Papilloma	Squamous-cell carcinoma	Other	
Benzo[ <i>a</i> ]pyrene	1.0	77	71.5	1	10	-	13.0
	1.7	88	68.6	-	25	-	28.4
	3.0	81	63.5	2	43	-	53.1
Carcinogenic PAH in cigarette-smoke condensate	6.3	84	67.3	-	27	1	32.1
	10.8	82	65.8	4	44	2	53.7
	18.9	92	57.3	1	65	1	70.7
Noncarcinogenic PAH in cigarette-smoke condensate	54.2	85	71.6	-	1	-	1.2
	162.6	92	69.9	-	2	-	2.2
	487.8	76	68.1	-	4	-	5.3
	1146.4	88	68.5	3	29	-	33.0
All PAH in cigarette smoke	60.5	87	64.2	1	28	1	32.3
	103.0	90	60.4	1	53	1	58.9
	181.6	97	51.3	2	68	1	70.1
Carcinogenic PAH in automobile-exhaust condensate	4.0	81	67.2	4	25	1	30.9
	6.8	88	67.1	3	53	1	60.2
	12.0	90	59.0	1	63	1	70.0

**Table 3. Induction of liver tumours in male Sprague-Dawley rats following administration of low levels of *N*-nitrosodiethylamine (NDEA), *N*-nitrosopyrrolidine (NPYR) and *N*-nitrosodiethanolamine (NDELA)**

Treatment (mg/kg)	Median total dose (mg/kg)	No. of animals	Median survival time (days)	Neoplasms of the liver (%)
Control	-	500	931	1
NDEA 0.1	58.4	80	854	45
NDEA 0.032	20.3	80	879	4
NDEA 0.01	6.3	80	914	4
NPYR 0.4	272	80	951	14
NPYR 0.133	85	80	897	3
NPYR 0.04	26	80	926	1
NDELA 2.0	1327	80	928	6
NDELA 0.63	421	80	934	5
NDELA 0.2	131	80	916	1
NDEA 0.032	21	100	914	12
NPYR 0.13	85			
NDELA 0.63	409			
NDEA 0.01	6.7	240	944	4
NPYR 0.04	27			
NDELA 0.2	135			
NDEA 0.0032	2.1	240	937	3
NPYR 0.013	8.7			
NDELA 0.063	42.4			

**Table 4. Combination experiments for syncarcinogenesis with different chemical carcinogens in rats and mice (total number of animals, 4374)**

Combination	Organotropism		Syncarcinogenesis
	Same	Different	
NDEA+DAB	+		+
NMOR+NDMA+ NDEA+DAB	+		+
C PAH	+		+
NDEA+DAST		+	0
NDEA+X-rays		+	0
DADP+X-rays		+	0
UR+DMBA		+	0

<sup>a</sup>NDEA, *N*-nitrosodiethylamine; NDMA, *N*-nitrosodimethylamine; NMOR, *N*-nitrosomorpholine; DAB, 4-dimethylaminoazobenzene; DAST, 4-dimethylaminostilbene; DADP, 4-dimethylaminodiphenyl; UR, urethane; DMBA, 7,12-dimethylbenz[*a*]anthracene; C PAH, carcinogenic polycyclic aromatic hydrocarbons

**Table 5. Effects of noncarcinogenic compounds or stimuli on chemical carcinogenesis in rats**

Carcinogen <sup>a</sup>	Target organ	Nonspecific measure	Syncarcinogenesis	Inhibition
NDEA	Liver	Ethyl alcohol	0	+
NPMA	Oesophagus	Ethyl alcohol	0	0
NBBA	Bladder	Cyclamate	0	0
NDEA	Liver	Immunosuppression, immunostimulation, stress, physical activity	0	0
ENU	Brain			
DMH	Intestine			
B[a]P	Subcutis			
CYCLO	All sites			
DMH	Intestine	Different diets	?	±
CYCLO	Bladder	Mesna	0	+
CYCLO	All sites	5-Fluorouracil, methotrexate	0	0
NDEA	Liver	Thioctic acid	0	0
ENU	Mammary gland	Nicotine	0	0
MNU	Intestine	Indomethacin	0	+
MNU	Mammary gland	Different diets	±	0
MPN	Oesophagus	Retinoids	0	0
NDEA	Liver	Disulfiram	0	+ <sup>b</sup>
NBBA	Bladder	Mesna	0	+

<sup>a</sup>NDEA, *N*-nitrosodiethylamine; NPMA, *N*-nitrosopentylmethylamine; NBBA, *N*-nitrosobutylbutanamine; ENU, *N*-ethyl-*N*-nitrosourea; DMH, 1,2-dimethylhydrazine; B[a]P, benzo[*a*]pyrene; CYCLO, cyclophosphamide; MNU, *N*-methyl-*N*-nitrosourea

<sup>b</sup>Protection against liver carcinogenesis but tumour induction in nasal cavity

With regard to *question (1)*: Additive carcinogenic effects were seen with agents with identical organotropism (Tables 1-3), including resorptive carcinogens with hepatotropic activity (*N*-nitroso compounds and 4-dimethylaminoazobenzene) and higher PAH, which occur in cigarette smoke and automobile exhaust condensate. The addition of carcinogenic effects was demonstrated by reduced induction times in combination experiments, even when the doses of the individual components were low. PAH-induced carcinogenesis was not modified by concomitant administration of noncarcinogenic PAH. These results confirm our thesis, since the mode of carcinogenic action in the target organ is identical or similar.

For *question (2)*: Concomitant administration of carcinogens with different organotropism did not result in additive carcinogenic effects. For this investigation, we chose compounds with hepatotropic activity (*N*-nitroso compounds) plus compounds with carcinogenic activity in the auditory meatus (aminostilbenes) in rats, and local carcinogens for the skin (7,12-dimethylbenz[*a*]anthracene) plus systemic carcinogens (urethane) in mice.

Additionally, X-rays were used in combination with hepatotropic carcinogens. The negative results obtained also corresponded to our thesis, in that different target cells are involved and addition of effects is not to be expected.

For *question (3)*: In the majority of experiments in which compounds with nonspecific toxic effects on a target organ were tested in carcinogenesis experiments, we observed no enhancing influence on the carcinogenic effect of the tropic carcinogen. Additional administration of ethanol, for instance, even inhibited liver carcinogenesis induced by low doses of *N*-nitrosodiethylamine (NDEA) (Habs & Schmähl, 1981). On the basis of recent investigations on the metabolism and activation of NDEA in the presence of ethanol (Schwarz *et al.*, 1980, 1984), this result appears plausible. It is obviously difficult to modulate carcinogenesis by nonspecific toxic factors. The design of such investigations must therefore be as quantitative as possible, and the toxic damage should not be so severe as to destroy the target cell, leaving scarred structures behind.

For *questions 4 and 5*: In principle, it should be possible to prevent carcinogenesis in some cases by administration of a specific antidote (Habs & Schmähl, 1983; Tacchi *et al.*, 1984). This was demonstrated in experiments on cyclophosphamide-induced urinary bladder carcinogenesis by concomitant administration of mesna (sodium 2-mercaptoethane sulfonate). To my knowledge, this is the first clear evidence of specific 'anticarcinogenesis', which has had practical clinical consequences. It would be extremely difficult, however, to modify carcinogenesis by nonspecific measures. Thus, for instance, no significant, practically relevant influence on carcinogenesis in the colon, oesophagus or mammary gland was seen after immunosuppression, immunostimulation or dietary variation, including high doses of retinoids. The effects observed were relatively small and nonspecific. In the case of dietary variation, caloric intake seems to play a more important role than specific dietary components.

Diverse results have been obtained with regard to combination effects. The most important findings derived from our data are the following:

(1) Addition of carcinogenic effects has to be assumed in principle, even with very low doses, when compounds of identical organotropism and cytotoxicity and with genotoxic activity are administered.

(2) The carcinogenic effects of compounds with different organotropism are not additive (Table 4).

(3) Carcinogenesis induced by genotoxic compounds cannot easily be modulated by nonspecific measures, which may or may not reach the target organ. Here, in particular, the doses of modulators have to be realistic (Table 5).

(4) Inhibition of carcinogenesis in certain target organs seems to be possible in principle. If they follow known metabolic pathways of carcinogens, carcinogenesis-related metabolites can be eliminated by specific antidotes. So far, this principle has worked in only a few cases.

It is obviously possible to protect an organ against carcinogenic activity, but the carcinogenic manifestation is then shifted to other tissues (Schmähl *et al.*, 1976c). This was first demonstrated by administration of *N*-nitrosodimethylamine and NDEA together with disulfiram: few hepatomas appeared in treated animals, but increased tumour rates were found in the oesophagus and nasal cavity — tumour types not seen after administration of the nitrosamines alone.

Combination effects are an essential problem in carcinogenesis to be tackled in the future. Experimental investigations should be designed to improve our basic knowledge; but, in individual cases, the possibilities of reducing carcinogenic effects by administering specific combinations should be investigated (Habs *et al.*, 1981; Berger *et al.*, 1983). It would be important not only to identify additive effects but also to find possibilities for inhibiting carcinogenesis, even though the available findings have only seldom resulted in practical application (Habs & Schmähl, 1983). Experimental investigations on combination effects must be strictly quantitative and must aim at the evaluation of the effects of low and very low doses, in order to reflect the human situation so that the ensuing results can play an important role in practical risk assessment.

# SYNCARCINOGENESIS AT LOW EXPOSURE LEVELS OF THREE NITROSAMINES WITH A COMMON TARGET OF CARCINOGENIC ACTION: PRELIMINARY RESULTS

M.R. Berger & D. Schmähl

*German Cancer Research Center, Im Neuenheimer Feld 280,  
6900 Heidelberg, Federal Republic of Germany*

The carcinogenic activities of *N*-nitrosodiethylamine (NDEA), *N*-nitrosopyrrolidine (NPYR) and *N*-nitrosodiethanolamine (NDELA) and of the combination of the three nitrosamines was determined in Sprague-Dawley rats at three logarithmically spaced exposure levels. The highest doses of the individual nitrosamines and of their combination, chosen so as to induce low specific incidences of liver tumours following life-long administration, resulted in median survival times for all treated groups of more than 30 months. The macroscopically determined incidences of liver tumours were 1% in control rats, 45%, 4% and 4% following administration of NDEA, 14%, 3% and 1% following administration of NPYR, 6%, 5% and 1% following administration of NDELA and 12%, 4% and 3% following the combined administration of all three nitrosamines. These results indicate linear, additive carcinogenicity with respect to their common target organ in the dose range investigated.

Extrapolation from experimental carcinogenicity studies in rodents to the human situation is difficult for three main reasons: (1) the doses used in experiments usually exceed human exposure levels by several orders of magnitude; (2) administration of single carcinogens does not permit evaluation of the possible in-vivo interactions of several agents at the same time — a situation encountered by humans; and (3) biological responses might differ in rodents and man. This study was designed as a contribution to solving the first two problems. It was intended to assess the syncarcinogenic action of combined administration to Sprague-Dawley rats of low doses of three nitrosamines which induce tumours in the same target organ. Exact dose-response curves for the individual agents and for the combinations were a necessary experimental prerequisite.

## Selection and administration of carcinogens

NDEA, NPYR and NDELA (kindly supplied by Dr R. Preussmann, Institute of Toxicology and Chemotherapy, German Cancer Research Center, Heidelberg, FRG) were chosen for this experiment, because the liver is known to be the main target organ of their carcinogenic activity and dose-response relationships have been calculated from previous experiments. These made it possible to extrapolate to the daily doses that induce low incidences of specific tumours after lifelong administration (Druckrey *et al.*, 1963; Preussmann *et al.*, 1977, 1982).

The three nitroso compounds were each administered daily at three dose levels five times per week in the drinking-water to groups of 80 male Sprague-Dawley rats. Groups of 100-240 rats received all three carcinogens in doses escalating by the same factor as in the

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groups receiving one carcinogen (i.e., 3.16), the medium dose of the individually-dosed groups being the highest dose in the combinations (Table 1). The experiment was started in rats  $100 \pm 6$  days old, and administration of the nitroso compounds was stopped after 39 months, when 90% of the animals had died. All rats were observed for life; dead animals were dissected to establish the specific cause of death; all tumours and macroscopically changed organs were recorded. These macroscopic findings served as the data base for the present evaluation, because the histological evaluation has not yet been finished.

**Table 1. Incidence of liver tumours in male Sprague-Dawley rats following oral administration of NDEA, NPYR and NDELA**

Treatment (mg/kg) <sup>a</sup>	No. of animals	Median total dose (mg/kg)	Median survival time (95% confidence interval) <sup>b</sup>	Standard significance <sup>c</sup>	Neoplasms of the liver <sup>d</sup>	
					No.	%
Control	500	-	931 (910 - 945)	-	5	1
NDEA 0.1	80	58.4	854 (816 - 892)	0.00001	36	45
0.032	80	20.3	879 (812 - 939)	0.0023	3	4
0.01	80	6.3	914 (879 - 1017)	0.504	3	4
NPYR 0.4	80	272	951 (854 - 1001)	0.44	11	14
0.133	80	85	897 (875 - 948)	0.074	2	3
0.04	80	26	926 (907 - 989)	0.895	1	1
NDELA 2.0	80	1327	928 (897 - 985)	0.32	5	6
0.63	80	421	934 (908 - 990)	0.91	4	5
0.2	80	131	916 (889 - 968)	0.394	1	1
NDEA 0.032 + NPYR 0.13 + NDELA 0.63	100	21 85 409	914 (875 - 983)	0.282	12	12
NDEA 0.01 + NPYR 0.04 + NDELA 0.2	240	6.7 27 135	944 (908 - 983)	0.726	9	4
NDEA 0.0032 + NPYR 0.013 + NDELA 0.063	240	2.1 8.7 42.4	937 (903 - 965)	0.455	6	3

<sup>a</sup>Administration in drinking-water

<sup>b</sup>Days from start of experiment

<sup>c</sup>Rank sum test according to the Kaplan-Meier estimate, without censoring of data

<sup>d</sup>Sum of macroscopically detected lesions

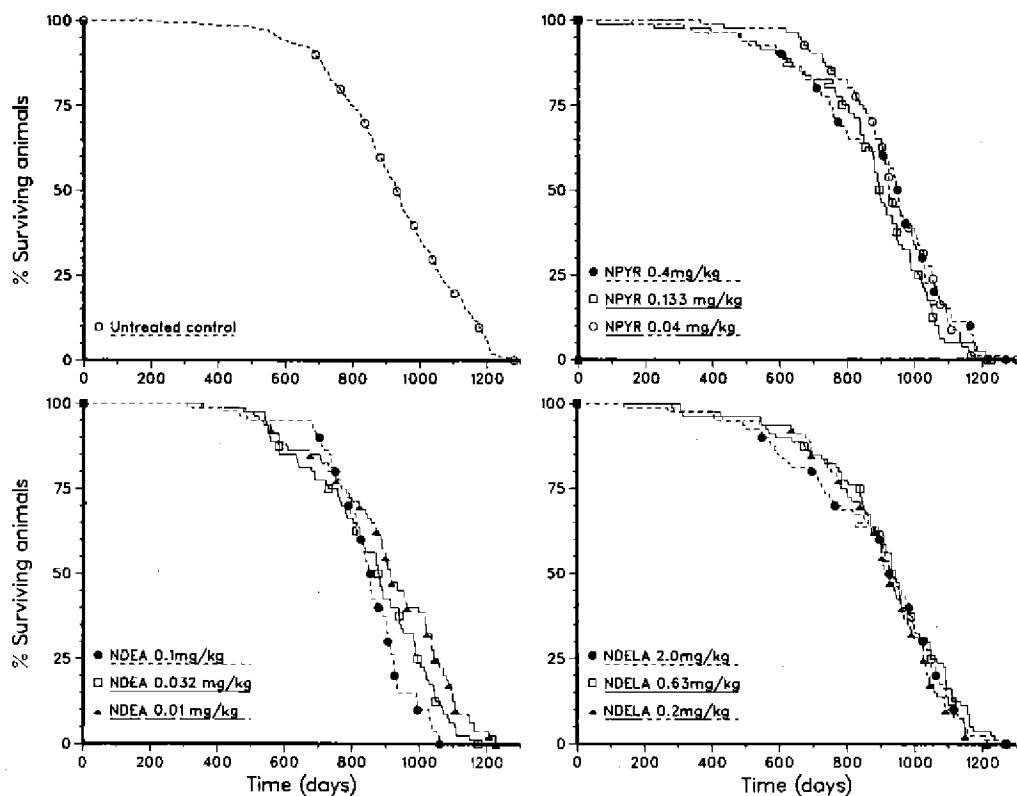
### Occurrence of liver tumours

The median survival of untreated controls was 34 months — a sufficiently long period to detect spontaneous liver tumours. Malignant or benign liver neoplasms were recorded in 1% of 500 control animals (Table 1); all occurred during the terminal stages of the experiment and would not have been detected if the experiment had been terminated after two years of administration, as is usually done in conventional carcinogenicity tests.

Administration of a median total dose of 58.4 mg/kg NDEA shortened the median life span by 2.5 months ( $p = 0.00001$ ) and gave rise to 45% of the liver tumours. Equally high yields of liver neoplasms were not found in rats receiving the medium (4%) or low dose (4%) of NDEA; the median survival time of the latter group was no different from that of untreated controls, whereas the medium dose significantly shortened survival ( $p = 0.0023$ ).

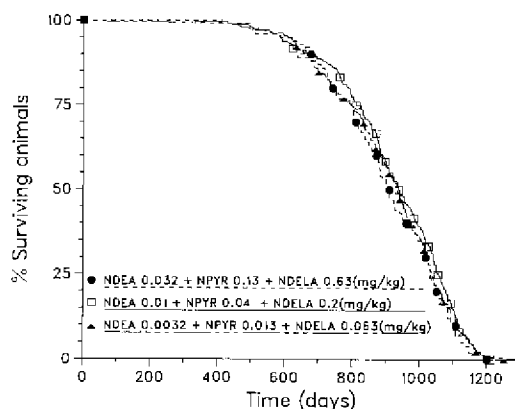
A less steep dose-response relationship for liver tumours was observed in rats that received NPYR or NDELA. The high dose of NPYR induced liver tumours in 14% of rats, the medium dose in 3% and the low dose in 1%; 6%, 5% and 1% of NDELA-treated animals had liver tumours in the three dose groups, respectively. The induction of tumours had no obvious influence on the survival times of the animals treated with the latter two carcinogens (Fig. 1) or with the combination of nitrosamines (Fig. 2), in which the incidences of liver tumours were 12% (high dose), 4% (medium dose) and 3% (low dose).

**Fig. 1. Percent survival of Sprague-Dawley rats in relation to time following oral administration of three logarithmically spaced dosages of NDEA, NPYR and NDELA in comparison to untreated controls**





**Fig. 2. Percent survival of Sprague-Dawley rats in relation to time following combined oral administration of NDEA, NPYR and NDELA at three logarithmically spaced dose levels**



### Implications of liver tumour occurrence

NDEA, NPYR and NDELA are mutagenic and carcinogenic at the usual experimental dosages, and these effects are causally related to interactions of nitrosamine-derived metabolites with DNA molecules (Preussmann & Stewart, 1984). Although no threshold is generally accepted for genotoxic compounds that would indicate a 'safe' level for human beings (Preussmann, 1980), a 'no-effect level' can be defined in experimental systems which is too low to allow the manifestation of tumours within the lifetime of the animal strain used.

Little is known about the validity of such 'quasi-thresholds' when exposure is to more than one genotoxic compound. One aim of this study was to contribute quantitative data on tumour yields following lifelong, concomitant

administration of three hepatotropic nitrosamines. Investigations on the dose-response relationships for the individual agents were included in this study as a necessary basis for a quantitative evaluation. The influence of variations due to ageing was minimized by selecting dosages that enabled survival in all treated groups to be comparable to that of controls. A clear dose-dependency of liver tumour formation was seen, even at very low levels of exposure to the individual agents. No shift in organotropism was observed. These findings support previous assumptions that 'safety margins' between the lowest active doses in animals and the average human exposure to these compounds are small (Schmähl, 1979, 1981; Preussmann, 1984). The incidence of liver tumours in animals that received the combination of NDEA, NPYR and NDELA indicate *linear additivity* in the dose range investigated. These results validate and extend semiquantitative investigations on the syncarcinogenic action of four hepatotropic carcinogens (Schmähl, 1970). Since an increased rate of liver tumours was seen even with the lowest dose of the combination — a rate twice that of untreated controls — the relevance of these concentrations, which approach the range of possible human exposure, has to be reappraised. Similar additivity in terms of tumour occurrence was found following concomitant administration of polycyclic aromatic hydrocarbons (Schmähl *et al.*, 1977). This parallel reinforces the importance of investigations on syncarcinogenic effects.

The origin of the liver tumours observed in control animals is not clear. Although a specific nitrosamine-poor diet was used (less than 2 ppm of either NPYR or NDMA), it is impossible to exclude completely contamination with nitrosamines in conventional standardized diets. Possible *in-vivo* formation of nitrosamines with carcinogenic organotropism to the liver also has to be taken into account. However, since the same diet was fed to all animals, similar influences would have been active in all experimental groups. The additional sources of nitrosamines, however, account for at most 1% of the total liver tumour yield and can therefore be ignored in view of the linear additivity in carcinogenic activity observed in the experimental groups subjected to combined administration of the three nitrosamines.

# LUNG CARCINOGENESIS BY N-NITROSOBIS(2-HYDROXYPROPYL)AMINE-RELATED COMPOUNDS AND THEIR FORMATION IN RATS

Y. Konishi<sup>1,3</sup>, Y. Yokose<sup>1</sup>, Y. Mori<sup>2</sup>, H. Yamazaki<sup>1</sup>, K. Yamamoto<sup>1</sup>,  
A. Nakajima<sup>1</sup> & A. Denda<sup>1</sup>

<sup>1</sup>Department of Oncological Pathology, Cancer Center,  
Nara Medical College, 840 Shijo-cho, Kashihara, Nara 634;  
and <sup>2</sup>Laboratory of Radiochemistry, Gifu Pharmaceutical University, 6-1,  
Mitahora-higashi 5-chome, Gifu 502, Japan

Lung carcinogenesis by a single intraperitoneal injection of *N*-nitrosobis(2-hydroxypropyl)amine (NDHPA) and related compounds was studied in male Wistar rats. NDHPA, *N*-nitrosomethyl(2-hydroxypropyl)amine (NMHPA), *N*-nitrosobis(2-oxopropyl)amine (NDOPA), *N*-nitroso(2-hydroxypropyl)(2-oxopropyl)amine (NHPOPA) and *N*-nitroso-2,6-dimethylmorpholine (NDMMOR) induced high incidences of lung neoplasms in rats. The formation of NDHPA, NDMMOR and NMHPA in the stomach of rats treated with precursor amines and sodium nitrite was detected by high-performance liquid chromatography (HPLC).

Tris- and bis(2-hydroxypropyl)amine (THPA and DHPA) are widely used in a variety of industries (The Chemical Daily Co., 1984). It has been reported that NDHPA is found in commercial samples of TPHA and DHPA and is possibly formed chemically from TPHA and nitrite under relatively mild conditions (Issenberg *et al.*, 1984), suggesting that there is a potential for human exposure. We have reported that lung carcinomas are induced by oral or intraperitoneal injection of NDHPA in rats (Konishi *et al.*, 1978a, 1979). In this paper, we report lung carcinogenesis by NDHPA and related compounds and the formation of these compounds in rats.

## Lung carcinogenesis by NDHPA and related compounds

Male Wistar rats (Shizuoka Laboratory Animal Center, Shizuoka, Japan), six weeks old, weighing approximately 180 g each, were used. NDHPA, NMHPA, NDOPA, NHPOPA and NDMMOR were synthesized by the method described previously (Mori *et al.*, 1984). Each compound, dissolved in saline, was injected intraperitoneally at the dose indicated in Table 1. All rats were fed a commercial stock diet (MF, Oriental Yeast Ind., Tokyo), then killed 55 weeks after treatment, completely autopsied and examined histologically. As shown in Table 1, each compound induced bronchiolo-alveolar and bronchial neoplasms in the lung; neoplasms were also seen in the thyroid, liver and kidney, however, their incidence was significantly lower than that of the lesions in the lung.

<sup>3</sup> To whom correspondence should be addressed.

**Table 1. Incidences of lung tumours in rats treated with NDHPA and related compounds**

Compound	Dose (mg/kg bw)	Effective no. of rats	Tumour incidence (%)					Bronchial region (papilloma)
			Bronchiolo-alveolar region <sup>a</sup>					
			Total	A	AC	ASC	S	
Saline		10	0	0	0	0	0	0
NDHPA	1000	10	6 (60)	4 (40)	3 (30)	1 (10)	0	6 (60)
	3000	10	9 (90)	7 (70)	8 (80)	4 (40)	0	7 (70)
NMHPA	45	9	2 (22)	0	1 (11)	0	1 (11)	1 (11)
	89	9	6 (67)	4 (44)	3 (30)	1 (11)	0	1 (11)
NDOPA	53	10	1 (10)	1 (10)	0	0	0	3 (30)
	106	10	10 (100)	10 (100) <sup>b</sup>	0	0	0	5 (50)
NHPOPA	212	10	6 (60)	5 (50)	2 (20)	1 (10)	1 (10)	0
	424	6	6 (100)	3 (50)	3 (50)	0	0	1 (17)
NDMMOR	110	12	0	0	0	0	0	2 (17)
	221	11	1 (9)	1 (9)	0	0	0	1 (9)
	442	9	4 (44)	2 (22)	1 (11)	1 (11)	0	1 (11)

<sup>a</sup>A, adenoma; AC, adenocarcinoma; ASC, adenosquamous carcinoma; S, squamous-cell carcinoma

<sup>b</sup>*p* < 0.05 compared with NDHPA (100) group

#### Formation of NDHPA and related compounds *in vivo*

THPA, DHPA and 2,6-dimethylmorpholine (DMMOR) were obtained from Tokyo Chemical Industry, Ltd, Tokyo, and tri- and diethanolamine (TELA and DELA) from Wako Pure Chemical Industries, Ltd, Osaka, Japan. Methyl(2-hydroxypropyl)amine (MHPA) was synthesized as described previously (Mori *et al.*, 1984). Rats were fasted overnight and gavaged with each amine (100  $\mu$ mol in 1 ml) and/or sodium nitrite 400  $\mu$ mol in 1 ml). After 30 min, the stomach contents were taken out with 20 ml 1.15% potassium chloride in the presence of 0.25 mg ascorbic acid and of  $\alpha$ -tocopherol (Norkus *et al.*, 1984) and were extracted with 2  $\times$  10 volumes of ethyl acetate. The combined extracts were evaporated to dryness below 40°C, taken up in 0.5 ml acetonitrile, clarified through a 0.45- $\mu$ m membrane filter (type TM-2P, Toyo Roshi Co., Ltd, Tokyo), and analysed by HPLC. For analysis of DELA, TELA or THPA, the products were cleaned up by thin-layer chromatography prior to HPLC analysis.

The calculated recoveries of the *N*-nitrosamines from stomach contents were in the range of 94-99%. As shown in Table 2, nitrosation from secondary amines was inversely proportional to the basicity of the precursor amine. The yields of *N*-nitrosodiethanolamine (NDELA) and NDHPA from DELA and DHPA plus nitrite were approximately 100 and 400 times greater than those from TELA and THPA, respectively. Administration of DMMOR, DHPA or MHPA alone failed to produce the *N*-nitrosamines. NHPOPA was detected in the 24-h urine of rats treated with DMMOR or DHPA plus nitrite as well as in rats treated with NDMMOR and NDHPA (Underwood & Lijinsky, 1982; Mori *et al.*, 1984).

**Table 2. Formation of *N*-nitrosamines in the stomach of rats treated with amines and nitrite<sup>a</sup>**

Amine	pKa	Nitrite	No. of rats	Estimated formation of <i>N</i> -nitrosamines in the stomach (mean ± SE)		
				<i>N</i> -Nitrosamine (μg)	Amine nitrosated (%)	
TELA	7.75	+	3	NDELA	7.65 ± 0.54	0.0571 ± 0.0069
THPA	7.86	+	3	NDHPA	1.87 ± 0.20	0.0115 ± 0.0022
DMMOR	8.38	-	2	NDMMOR	< 4	
		+	2	NDMMOR	1151 ± 137	8.00 ± 0.95
DELA	8.85	+	3	NDELA	823 ± 34	6.14 ± 0.25
DHPA	8.97	-	2	NDHPA	< 3	
		+	3	NDHPA	651 ± 46	4.02 ± 0.28
MHPA	9.43	-	2	NMHPA	< 3	
		+	3	NMHPA	197 ± 5	1.66 ± 0.04

<sup>a</sup>Rats were fasted overnight before treatment; 30 min after intragastric administration of 100 μmol amine and/or 400 μmol nitrite, stomach contents were taken out with 1.15% potassium chloride, extracted with ethyl acetate in the presence of ascorbic acid and α-tocopherol and analysed for *N*-nitrosamines by HPLC.

# A POSSIBLE MECHANISM FOR THE DOSE-RESPONSE RELATIONSHIP OBSERVED FOR RENAL MESENCHYMAL TUMOURS INDUCED IN THE RAT BY A SINGLE DOSE OF *N*-NITROSODIMETHYLAMINE

H.E. Driver<sup>1,4</sup>, I.N.H. White<sup>1</sup>, F.S. Steven<sup>2</sup> & W.H. Butler<sup>3</sup>

<sup>1</sup>*MRC Toxicology Unit, Woodmansterne Road, Carshalton, Surrey;*  
<sup>2</sup>*Department of Biochemistry, University of Manchester, Manchester;*  
*and* <sup>3</sup>*British Industrial Biological Research Association (BIBRA),*  
*Woodmansterne Road, Carshalton, Surrey, UK*

The incidence of renal mesenchymal tumours induced in rats by *N*-nitrosodimethylamine (NDMA) is related to the dose in a sigmoidal dose-response curve. Each kidney bears only one or two tumours at 20-24 months. In contrast, one week after dosing, a large number of preneoplastic proliferative foci is present, the incidence of which is linearly related to dose and directly proportional to methylation of DNA by NDMA. It is suggested that most of these foci are removed by host defense mechanisms before they can progress to tumour, thus accounting for the sigmoid shape of the dose-response curve for the tumours.

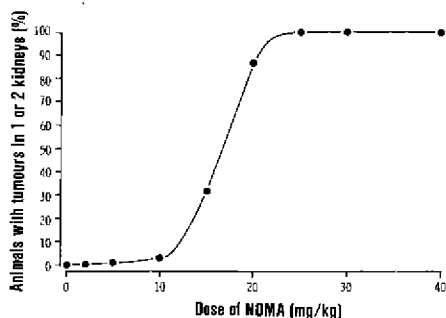
The hypothesis that chemical carcinogens induce tumours as a consequence of the binding of reactive metabolites to cellular DNA is widely accepted, and, because this binding is linearly related to dose, it is often assumed that the tumour incidence is directly proportional to the amount of alkylation and that no threshold can exist for carcinogenesis. However, it appears from both epidemiological and experimental work that thresholds do exist (Driver & McLean, 1986) and that the dose-response curves for tumours are frequently not linear. These experiments use NDMA-induced renal mesenchymal tumours as a model for the study of dose-response relationships in carcinogenesis.

## Induction of renal mesenchymal tumours

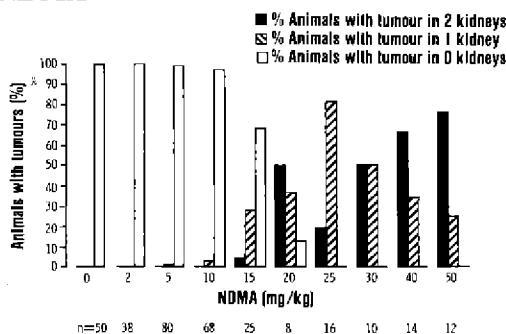
Swann and McLean (1968) showed that when rats are fed on a protein-free diet for three days prior to dosing with a single intraperitoneal dose of NDMA, they are protected from the lethal effects and survive to develop renal neoplasia. The proportion of epithelial and of mesenchymal tumours is dependent on the age of the rats at the time of dosing (Hard, 1979). In these experiments, weanling Fischer 344 rats were fed on sucrose for three days, then given a single injection of 2-50 mg/kg NDMA. The majority of tumours were of mesenchymal origin, as described by Hard and Butler (1970), and their incidence was related to the dose of NDMA in a sigmoid dose-response curve (Fig. 1). The number of tumours per kidney is small, most animals having only one or two tumours in each kidney at 20-24 months. The number of tumours per kidney is also dose-dependent (Fig. 2).

<sup>4</sup> To whom correspondence should be addressed

**Fig. 1. NDMA-induced renal mesenchymal tumours in rats at 20-24 months: dose-response relationship**



**Fig. 2. Incidence of renal mesenchymal tumours in rats at 20-24 months after treatment with NDMA**



### Induction of early proliferative foci of mesenchymal cells

Within a few days of dosing, a large number of small proliferative foci appears in mesenchymal cells in the renal cortex, usually associated with a glomerulus or a blood vessel. The number of these foci reaches a peak at three weeks and thereafter declines, so that at 12 weeks the kidneys appear normal, except for the development of one or two mesenchymal tumours per kidney at the higher doses.

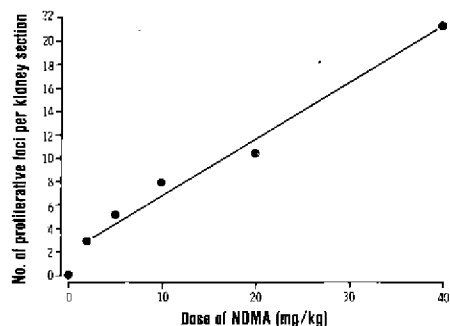
The identity of these lesions as putative pre- or early neoplastic foci is suggested by their cellular morphology and by their staining characteristics. All the tumours examined contained areas of alcian-blue-positive mucin and also possessed the enzyme guanidinobenzotase. This proteolytic enzyme is expressed by many tumours in man and animals. It can be demonstrated in sections by the use of 9-aminoacridine and propidium iodide as fluorescent probes, which are selective for the active centre of guanidinobenzotase. Increasing the concentration of 9-aminoacridine enhances the fluorescent signal due to molecular stacking.

The early proliferative foci of mesenchymal cells were similarly found to be positive for these two markers and have thus been quantified. Figure 3 shows the number of proliferative foci per single longitudinal section of kidney at different dose levels of NDMA three weeks after dosing. The number of foci is linearly related to the dose between 2 and 40 mg/kg.

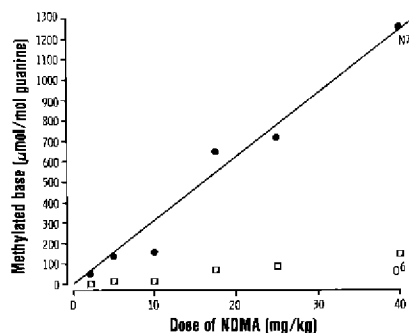
### Methylation of DNA by NDMA

Methylation of DNA at the *O*<sup>6</sup> and *N*<sup>7</sup> positions of guanine was measured 18 h after dosing using <sup>14</sup>C-NDMA. Methylation was also shown to be linearly related to dose between 2 and 40 mg/kg NDMA (Fig. 4).

**Fig. 3. NDMA-induced mesenchymal proliferative foci in rats after three weeks: dose-response relationship**



**Fig. 4. Methylation of kidney DNA by NDMA: dose-response relationship**



### Discussion and conclusions

The sigmoidal shape of the dose-response curve for NDMA-induced renal mesenchymal tumours demonstrates that tumour incidence is not linearly related to dose and that there may be an effective threshold: no tumour was induced at less than 5 mg/kg NDMA, despite the use of relatively large numbers of animals per group ( $n = 80$ ). However, the number of small foci of mesenchymal proliferation that appears shortly after dosing *is* linearly related to dose, and even with 2 mg/kg NDMA, which induces no tumours, there is a significant number of foci. The number of these early lesions is directly proportional to the amount of methylation of DNA: no threshold operates at this stage. Therefore, the shape of the dose-response curve for the tumours is a consequence of biological mechanisms operating to remove the vast majority of the early lesions, leaving only a few to progress to tumour. The exact nature of this host defence mechanism remains to be elucidated, but histological findings suggest that it involves an immunological reaction. Spontaneous regression may also be occurring.

Thus, dose-response relationships may exist in chemical carcinogenesis which are independent of the initial reaction with DNA.

### Acknowledgements

The authors would like to thank Mr R.J. Hill for skilled technical assistance and Dr V. Cunningham for advice on interpretation of the data.

# VASCULAR CHANGES AND LIVER TUMOURS INDUCED IN MINK BY HIGH LEVELS OF NITRITE IN FEED

N. Koppang<sup>1,3</sup> & A. Helgebostad<sup>2</sup>

<sup>1</sup>National Veterinary Institute, PO Box 8156 Dep, N-0033 Oslo 1; and

<sup>2</sup>Norwegian College of Veterinary Medicine, PO Box 8146 Dep,  
N-0033 Oslo 1, Norway

Two groups of female mink were fed a diet supplemented with 30-50 mg/kg bw sodium nitrite for up to six years. The first group also received dimethylamine hydrochloride. Seven male offspring from litters born in the first year were fed the same diet for nine months but showed no pathomorphological change. After three years on trial, female mink developed occlusive changes in some branches of the efferent hepatic veins, and 21% of the mink in group 1 and 31% in group 2 developed liver haemangioendotheliomas or precancerous liver changes. The pathomorphological changes were identical to those seen in animals exposed to *N*-nitrosodimethylamine (NDMA). This result indicates *in-vivo* formation of NDMA as a result of the high nitrite in the diet. However, NDMA was not measured in the blood of the nitrite-exposed mink.

Evidence that NDMA can be formed during the processing of feeds and food and cause malignant liver disease in cattle, sheep and fur-bearing animals was established by our experimental work in the early 1960s (Koppang, 1962; Ender *et al.*, 1964; Koppang, 1964; Koppang & Slagsvold, 1964; Koppang *et al.*, 1964; Sakshaug *et al.*, 1965; Koppang, 1966; Koppang & Helgebostad, 1966; Koppang, 1970). When nitrite preservation of raw fish was found to result in formation of NDMA during processing, we predicted that NDMA might occur in all human food in which nitrite was used as a curing agent and heated (Koppang & Slagsvold, 1964; Sakshaug *et al.*, 1965). Further experiments showed that NDMA was formed not only during processing but also during storage, the amines present reacting chemically with nitrite. Excess nitrite in fish meal increased the level of NDMA from 70 to 125 ppm (Koppang, 1974a,b). The main source of NDMA in fish meal is endogenous trimethylamine oxide (Koppang, 1974c). The trimethylamine oxide content increases with growth, varies within species and with the season and is highest during the winter (Shewan, 1961; Tokunaga, 1970).

As mink are quite susceptible to the toxic and carcinogenic effects of NDMA and have a very low tumour rate with normal feeding, we designed a long-term mink feeding experiment. The animals were fed an excess of nitrite with and without dimethylamine hydrochloride. For comparison, two further groups were fed the same basal diet to which was added either NDMA (Koppang & Helgebostad, 1976a) or NDMA from herring meal (Koppang & Helgebostad, 1987b).

## Material and methods

The experiments were carried out at the Research Farm for Fur Bearing Animals of the Norwegian College of Veterinary Medicine. Thirty female mink, nine months of age, were

<sup>1</sup> To whom correspondence should be addressed



used in the two experimental groups. These were mated, and seven of the males in the first litters were added to the experimental group of their mothers; these offspring were kept for only nine months and then sacrificed. The control group consisted of 20 female mink. The constituents of the basic feed are shown in Table 1.

**Table 1. Composition of basic feed**

Component	Percent	Protein (%)	Fat (%)	Carbohydrates (%)
Fish and fish offal	60	8.2	1.6	
Slaughter house offal (viscera)	26	3.0	3.0	
Precooked, coarse milled wheat	11	0.9	0.3	5.6
Vitamin mixture	3			1.0
Iron supplement, 5 mg				
Total	100	12.1	4.9	6.6
Kilocalories/kg		54.5	45.5	27
Percent energy/kg		43	36	21

Each mink in group 1 was also given 30 mg/kg bw sodium nitrite and 60 mg/kg bw dimethylamine hydrochloride on six days per week. Mink in group 2 were given 30 mg/kg bw per day sodium nitrite. After two years, the mink in groups 1 and 2 received 50 mg/kg bw per day sodium nitrite and the concentration of dimethylamine hydrochloride given to group 1 was increased to 100 mg/kg bw per day. The compounds were dissolved in water and mixed thoroughly into the basic diet six days per week; on Sundays, all mink were fed the same basic diet as the control group. The sodium nitrite (analytical grade) came from Merck (Darmstadt) and the dimethylamine hydrochloride from Fluka (38960).

Serum proteins were determined by electrophoresis, and haemoglobin concentrations were measured at intervals. All mink were autopsied and the kidney, heart, spleen and liver samples fixed in 10% formalin, paraffin embedded, sectioned and stained as reported previously (Koppang *et al.*, 1964).

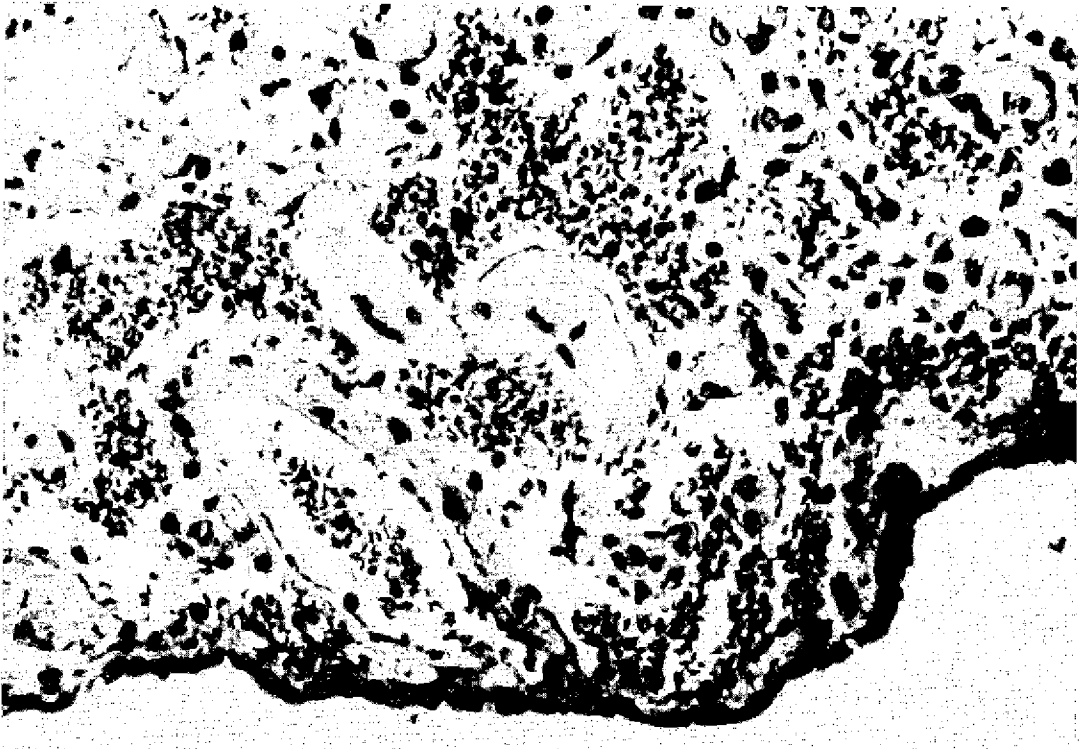
## Results

The treatment caused increased water intake, and the general health of the mink in the two treated groups was worse than that of the controls; two females in group 2 died from nonspecific diseases during the first year. The experiment was terminated after six years, when the three remaining females in each group were sacrificed.

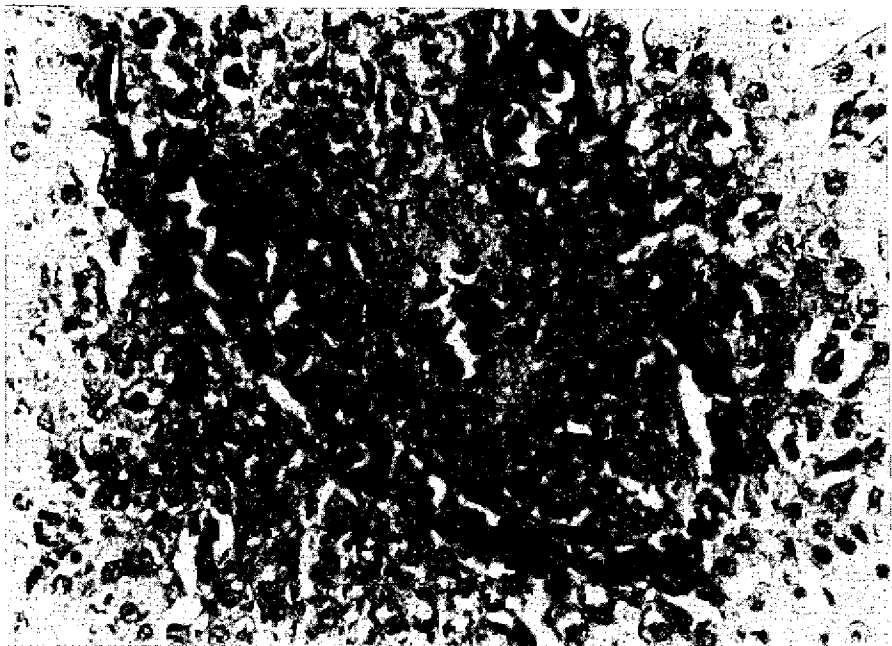
### *Pathomorphological changes*

*Group 1:* Two mink died at 53 and 69 months from abdominal haemorrhage due to rupture of liver haemangioendotheliomas (Fig. 1); one of these animals also showed myocardial infarcts. One mink sacrificed after six years on the experiment displayed precancerous changes of the liver. Three showed cancerous changes, giving a tumour frequency of 21%. The remaining 11 females died of metritis, plasmacytosis and nonspecific diseases. All mink that were on trial for more than three years had occlusive changes in some branches of the efferent hepatic veins (Fig. 2). The seven male offspring sacrificed after nine months of experimental feeding were all normal.

**Fig. 1. Liver of mink fed sodium nitrite and dimethylamine hydrochloride for life**



**Fig. 2. Liver of mink fed sodium nitrite and dimethylamine hydrochloride for life**



Occlusive changes in a hepatic vein. H & E  $\times 200$

*Group 2:* Two animals in this group died from ruptured liver tumours (haemangio-endotheliomas); two other animals that showed precancerous liver changes died from cachexia. A fifth animal, apparently healthy at the time of sacrifice at six years, had multiple haemangioendotheliomas in the liver lobes. Five mink of the total of 16 had liver tumours, giving a tumour frequency of 31%. The other 11 females died from nonspecific diseases. All mink on trial after three years had occlusive changes in some branches of the efferent hepatic vein. The seven male offspring sacrificed after nine months on trial were all normal.

*Group 3:* None of the control animals showed changes in the efferent hepatic veins, and no liver tumour was found.

## Discussion

Nitrosamines are formed by chemical reaction between nitrosating agents and nitrosatable amines, mostly secondary and tertiary amines. Probably the most important nitrosating agent for nitrosamine formation is nitrous anhydride, which forms readily from nitrite in aqueous acidic solution (Scanlan, 1983). Trimethylamine oxide in fish and nitrite used as a preservative or nitrogen oxide formed by drying directly over fires were the two precursors for NDMA formation in herring meal (Koppang, 1974c). Mink developed haemorrhagic liver necrosis and occlusive changes of the efferent hepatic vein when exposed to more than 0.2 mg NDMA/kg bw per day; at 0.2 mg/kg or slightly less, about 100% of the animals developed haemangioendotheliomas after a total uptake of 25-77 mg/kg bw (Koppang & Rimeslatten, 1976). The LD<sub>50</sub> for NDMA in mink is 7 mg/kg bw (Koppang & Helgebostad, 1987a), compared with 40 mg/kg bw for rats (Magee & Barnes, 1967).

Since the tumour frequency in group 1 was lower than that in group 2, addition of dimethylamine does not appear to increase NDMA formation, although the high level of nitrite fed to the treated groups facilitated reactions between nitrite, trimethylamine oxide and other amines from the fish and meat offal in the food. The amount of nitrite added to the feed of treated groups was 75-150 times higher than that accepted by the Food and Agriculture Organization and World Health Organization of the United Nations for daily intake. The pathomorphological changes seen in animals in groups 1 and 2 were comparable to those found in a long-term feeding experiment in which mink received 0.025 mg/kg bw per day NDMA (Koppang & Helgebostad, 1987b). The amount of trimethylamine oxide available was high for all groups, since the feed contained 60% fish products. Lijinsky *et al.* (1972) indicated that tertiary amines react with nitrite at pH 3-6.5, a range that includes the pH of mink stomach.

The tumours seen in the liver were typical of those found in mink exposed to NDMA (Koppang, 1966; Koppang & Rimeslatten, 1976; Koppang & Helgebostad, 1987a,b). Tumours of the same type have been described in other laboratory animals exposed to nitrosamines (Wayss *et al.*, 1979).

NDMA-induced occlusive changes were seen regularly in areas of the hepatic vein in mink in groups 1 and 2 (Fig. 2). Dilatation of the sinusoids with irregular nuclear enlargement of the endothelium is the next step in the development of haemangio-endothelioma. Like Wayss *et al.* (1979), we considered these changes to be precancerous. The occlusive changes in some areas of the efferent hepatic veins, the precancerous changes in the sinusoids and the development of haemangioendotheliomas in the liver during the six-year experimental period strongly indicate that NDMA is the causative factor. Ishiwata *et al.* (1981) showed that nitrate injected into the lower digestive tract of rats was rapidly reduced to nitrite by the contents. After simultaneous injection of 250 µg each of nitrate and dimethylamine into ligated sections, *N*-nitrosodiethylamine was formed at levels of 10-51 ppb. Rapid absorption of NDMA from the sections was also observed.

Continuous feeding to rats of 1 ppm NDMA in the diet has been suggested to be a threshold dose (Terracini *et al.*, 1967). More than 0.2 mg/kg bw per day NDMA in the diet of sheep (Koppang, 1974a), cattle (Koppang, 1974b), mink (Koppang & Rimeslåtten, 1976; Koppang & Helgebostad, 1987a,b) and foxes (Koppang *et al.*, 1981) caused toxic hepatitis and death after a total intake of 18-50 mg/kg bw NDMA. In experiments with ruminants (Koppang, 1974a,b), we saw focal occlusive changes in branches of the hepatic veins in animals consuming 0.1 mg/kg bw per day NDMA over a longer period. In mink, daily doses of 0.025 mg/kg over a two- to three-year period produced changes in some areas of the efferent hepatic veins. With continuous low exposure, dilatation of the sinusoids with some nuclear enlargement of the covering endothelium occurred. These 'precancerous' liver changes developed with time into liver haemangioendotheliomas, comparable with the changes observed in groups 1 and 2. Similar changes are observed after exposure of other domestic animals to NDMA (Koppang, 1980, 1981; Koppang *et al.*, 1981; Koppang, unpublished data). If it had been possible to measure NDMA in blood, as reported by Gough *et al.* (1983), we would have been able to give a direct answer to the question: Did the high level of nitrite in the feed of groups 1 and 2 serve as a precursor of NDMA and thus cause the observed vascular changes and liver tumours? Unfortunately, at the time of our experiment, this technique was not available.

In the two decades since we described a malignant liver disease in fur-bearing and ruminant animals caused by toxic herring meal produced from nitrite-preserved herring, much research has been carried out on the toxic and carcinogenic effects of NDMA. To date, no threshold value below which no damage from NDMA occurs has been determined: at a daily dose of 0.025 mg/kg bw NDMA, a number of mink died from nonspecific disease before accumulating a carcinogenic dose of NDMA.

From a pathomorphological point of view, the greatest difference between animal and human pathology is the degree of vessel changes. The damaging effects of NDMA on the vessels include not only those on the hepatic veins, which appear to be responsible for liver cirrhosis and ascites, but also those on vessels in the heart, kidneys and other organs. In the present experiment, acute thrombosis of the heart, kidneys and liver occurred in some of the mink in groups 1 and 2, as seen previously in mink, foxes and dogs after exposure to NDMA. One of our blue foxes died suddenly from brain haemorrhages during a NDMA experiment (Koppang *et al.*, 1981). The vessel changes induced by NDMA may be as important as the tumorigenic changes; they also develop after low daily exposures and are the first recognizable histological changes. Vascular tumours of the type found in these experiments have been reported to occur with greater frequency in humans (Wayss *et al.*, 1979), indicating further research.

# ZINC-DEFICIENCY AND THE DEVELOPMENT OF MALIGNANT LYMPHOMA IN RATS GIVEN A SINGLE INTRAGASTRIC DOSE OF *N*-METHYL-*N*-NITROSOUREA

L.Y.Y. Fong<sup>1,3</sup>, C.P. Lui, L. Ma-Tung<sup>2</sup> & W.L. Ng<sup>2</sup>

<sup>1</sup>*Department of Biochemistry and* <sup>2</sup>*Department of Pathology, Faculty of Medicine, University of Hong Kong, Hong Kong*

Zinc-deficient rats that had received a single dose of *N*-methyl-*N*-nitroso-urea (MNU) intragastrically developed malignant lymphomas involving the liver, spleen, lung and kidney, as well as the usual epithelial tumours at the site of administration. Interestingly, the incidence of squamous-cell carcinoma of the pharynx was significantly higher in zinc-deficient rats than in control animals. Since MNU does not require metabolic activation, the development of the malignant lymphomas might be related to the generally depressed immunological state of zinc-deficient rats.

We demonstrated earlier that nutritional zinc deficiency enhances and modifies the tumorigenic activities of nitrosamines in rats. A significantly higher incidence of oesophageal tumours was observed in zinc-deficient rats given *N*-nitroso-*N*-methylbenzylamine, an oesophagus-specific carcinogen, than in zinc-sufficient counterparts (Fong *et al.*, 1978). Furthermore, *N*-nitrosodimethylamine, a liver carcinogen, was tumorigenic to the forestomach of zinc-deficient rats (Fong *et al.*, 1984). In order to examine whether dietary zinc deficiency has a modifying effect on the tumorigenic action of a directly-acting carcinogen that does not require metabolic activation, the carcinogenic effect of a single intragastric dose of MNU was studied in zinc-deficient rats.

## Experimental plan

Sixty weanling male Sprague-Dawley rats were used: 30 were fed a zinc-deficient diet containing 10 ppm zinc, and 30 were pair-fed a zinc-sufficient diet containing 100 ppm zinc. The composition of the diet has been described previously (Fong *et al.*, 1978). After five weeks on these diets, each animal received a single intragastric dose of MNU at 30 mg/kg bw. Rats were killed when they became moribund, and all rats were killed at the 75th week. Complete necropsies were performed; the major organs were fixed in 10% neutral buffered formalin and processed for histological examination.

## Development of malignant lymphomas in zinc-deficient rats

None of the pair-fed zinc-sufficient control rats developed a malignant lymphoma, but 13 of the zinc-deficient rats developed malignant lymphomas involving the spleen, liver, kidney and lung (Table 1). The distribution was as follows: nine rats developed malignant lymphomas in the spleen, two rats in the spleen and liver, one rat in the spleen, liver and kidney, and one rat in the lung.

<sup>3</sup> To whom correspondence should be addressed

**Table 1. Malignant lymphomas in zinc-deficient rats given a single intragastric dose of MNU**

Organ	No. (%) of rats with malignant lymphomas
Spleen	12 (41)
Liver	3 (10)
Lung	1 (3)
Kidney	1 (3)

of an indirect carcinogen like *N*-nitrosodimethylamine is not present with a direct carcinogen like MNU. The interesting observation that nine zinc-deficient rats and only one zinc-sufficient control developed squamous-cell carcinoma of the pharynx awaits further studies.

**Table 2. Lesions other than lymphomas in zinc-deficient and zinc-sufficient rats given a single intragastric dose of MNU**

Lesion	No. of rats (%)	
	Zinc-deficient	Zinc-sufficient
<b>Stomach</b>		
Squamous papilloma	21 (72)	20 (69)
Squamous carcinoma	3 (10)	5 (17)
Adenocarcinoma	3 (10)	4 (13)
Leiomyosarcoma	0	1 (3)
<b>Pharynx</b>		
Squamous carcinoma	9 (31)	1 (3)
<b>Kidney</b>		
Nephroblastoma	0	1 (3)
<b>Salivary gland</b>		
Adenocystic carcinoma	0	1 (3)
<b>Breast</b>		
Tumour	1 (3)	0
<b>Skin</b>		
Adenocarcinoma	1 (3)	0

Other lesions were also found in both dietary groups (Table 2), which were mostly single or multiple squamous papillomas or malignant tumours at the junction of the fore- and hindstomach. The concurrent development of gastric tumours was to be expected because of the route of administration of MNU. However, no significant difference in the incidences of gastric tumours was observed between the two groups, in contrast to the results of our previous study with *N*-nitrosodimethylamine, a carcinogen which requires metabolic activation (Fong *et al.*, 1984). This suggests that the modifying effect of zinc deficiency on the tumorigenic activity

MNU has been shown to be tumorigenic in a variety of tissues of several animal species, depending on the dose and route of administration (Druckrey *et al.*, 1967). For instance, the incidence of neuroglial tumours was greatest when MNU was given intravenously at low weekly doses; increasing individual doses resulted in greater incidences of extraneural tumours (Swenberg *et al.*, 1975). In the present study, with a single oral dose of 30 mg/kg MNU, zinc-sufficient rats developed tumours in the stomach (69%), kidney (3%) and salivary gland (3%). With a much higher single oral dose, 90 mg/kg, Leaver *et al.* (1969) found high incidences of tumours in the stomach and kidney, consistent with the expected action of a high single oral dose of the compound.

The most notable finding of this study is the development of malignant lymphomas in zinc-deficient rats (Table 1). The Sprague-Dawley rat has no history of naturally occurring malignant lymphomas (MacKenzie & Garner, 1973). Other investigators have demonstrated the induction of thymic lymphomas with multiple doses of MNU: with biweekly intragastric applications of MNU (20 mg/kg twice weekly for nine weeks), Koestner *et al.* (1977) induced a 100% incidence of thymic lymphomas and gastric carcinomas in these animals. All but two lymphomas were restricted to the thymus; of these (both histiocytic lymphomas), one involved the spleen and liver and the other infiltrated locally into cervical and thoracic regions.

While thymic lymphomas have been induced experimentally in rats with agents other than MNU (Suzuki, Y. *et al.*, 1984), malignant lymphomas of B-cell origin are rarely observed in rats. Since MNU does not require prior enzymic activation for its carcinogenic activity, the development of malignant lymphomas at sites other than the thymus in zinc-deficient rats could very well be related to the nutritional state of the animal. In experimental animals, including rats, mice and monkeys, zinc deprivation results in a variety of aberrant immunological parameters, including impaired in-vitro blast transformation of lymphocytes in response to B- and T-cell mitogens (Gross *et al.*, 1979; Beach *et al.*, 1983; Haynes *et al.*, 1985), and abnormal levels of immunoglobulin profile (Beach *et al.*, 1982). Moreover, it has been demonstrated that zinc deficiency in children can lead to alterations in immunocompetence, as manifested by reduced cell-mediated immunity (Golden *et al.*, 1977, 1978). It appears possible that the malignant lymphomas induced in zinc-deficient rats in the present study could be related to the impaired immune functions brought about by nutritional zinc deficiency.

#### **Acknowledgements**

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# INFLUENCE OF ETHYL ALCOHOL ON CARCINOGENESIS INDUCED BY VOLATILE *N*-NITROSAMINES DETECTED IN ALCOHOLIC BEVERAGES

L. Griciūtė<sup>1</sup>, M. Castegnaro<sup>2</sup> & J.C. Béréziat<sup>2</sup>

<sup>1</sup>*Oncological Institute of Lithuanian SSR, Vilnius USSR; and*

<sup>2</sup>*International Agency for Research on Cancer, Lyon, France*

Endemic oesophageal cancer has been related to the consumption of alcoholic beverages in some areas of the world. Volatile *N*-nitrosamines — *N*-nitrosodimethylamine (NDMA), *N*-nitrosodiethylamine (NDEA) and *N*-nitrosodipropylamine (NDPA) — have been detected in 20-50% of samples of coloured alcoholic beverages, including apple brandy and whisky (Tuyns *et al.*, 1980). It is not yet known whether the small quantities of *N*-nitrosamines in alcoholic beverages can induce oesophageal cancer in humans.

Some data indicate that the carcinogenicity of certain chemicals may be increased by ethanol (Horie *et al.*, 1965; Gibel, 1967; McCoy *et al.*, 1982; Litvinov *et al.*, 1986); however, the results obtained by other laboratories have been contradictory (Schmähl *et al.*, 1965; Schmähl, 1966; Gurkalo & Zabezhinsky, 1982; McCoy *et al.*, 1982). A study was performed at IARC on groups of mice given small doses of different *N*-nitrosamines either in water or in ethanol solution (Griciūtė *et al.*, 1981, 1982, 1984). The present paper deals with the results of an experiment in which mice were given three *N*-nitrosamines simultaneously.

A total of 628 C57Bl male and female mice were divided into nine groups, odd-numbered groups receiving treatment in 40% ethanolic solutions and even-numbered groups in aqueous solutions (Table 1). Groups I-VI received intragastric instillations of 0.03 mg NDMA, NDEA or NDPA twice a week for 50 weeks, while groups VII and VIII received 0.01 mg of each *N*-nitrosamine. Group IX received only ethanol. Animals were killed after 79 weeks and the internal organs were fixed in formalin, prepared for microscopy and examined histologically.

The results for groups I-VI have been described previously (Griciūtė *et al.*, 1981, 1982, 1984). In the present experiment, the most common tumours were of the forestomach (or oesophagus) and of the lungs. Malignant forestomach tumours were seen in 50% of animals in group VII and in 11% of animals in group VIII; no such tumour occurred in controls ( $p < 0.00005$ ). Pulmonary adenomas were detected in 78% of mice in group VII, 48% in group VIII and 6% in group IX. In addition, two aesthesioneuroblastomas infiltrating the brain were detected in mice in group VII. The incidences of other tumours were not significant.

In this experiment, the presence of ethanol as a solvent for the three *N*-nitrosamines enhanced the development of both benign and malignant tumours of the forestomach, and of lung tumours. The comparable incidences of forestomach tumours in animals treated with all three *N*-nitrosamines (groups VII and VIII) and in those given NDEA or NDPA alone (groups III and IV, and V and VI) implies a syncarcinogenic effect of these *N*-nitrosamines. This effect is even greater for the induction of pulmonary adenomas (group VII *versus* group III and *versus* group V). The number of lymphomas was similar to that observed with NDEA or NDPA alone and much greater than with NDMA alone.



Table 1. Carcinogenicity to C57Bl male and female mice of treatment with *N*-nitrosamines in ethanol

Treatment (dose per mouse)	No. of animals <sup>a</sup>	No. (%) of animals with tumours	Fore-stomach		Pulmonary adenomas	Hepatomas	Lymphomas	Aesthesio-neuro-blastomas	Other tumours	
			Benign	Malignant					Benign	Malignant
I NDMA (3 mg) + ethanol	70 (66)	48 (72)	-	-	2	28 (42)	4 (6)	24 (36)	-	-
II NDMA (3 mg) + water	70 (66)	43 (65)	-	-	-	40 (60)	8 (12)	-	-	-
III NDEA (3 mg) + ethanol	70 (69)	68 (98)	21 (30)	32 (46)	46 (67)	6 (9)	21 (30)	-	1 (1.5)	9 (13)
IV NDEA (3 mg) + water	70	68 (97)	31 (44)	7 (10)	48 (69)	15 (21)	45 (64)	-	3 (4)	-
V NDPA (3 mg) + ethanol	70	68 (97)	29 (41)	36 (51)	20 (29)	6 (9)	18 (26)	-	-	-
VI NDPA (3 mg) + water	70	48 (69)	2 (34)	7 (10)	9 (13)	4 (5)	12 (17)	-	9 (13)	2 (3)
VII NDMA, NDEA, NDPA (1 mg) + ethanol	70	69 (98)	24 (34)	35 (50)	55 (78)	3 (4)	23 (33)	2 (3)	2 (3)	1 (1.5)
VIII NDMA, NDEA, NDPA (1 mg) + water	70	61 (87)	17 (24)	8 (11)	34 (48)	7 (10)	32 (45)	-	-	-
IX Ethanol	68	21 (31)	3 (4)	-	4 (6)	1 (1)	13 (19)	-	4 (5)	3 (4)

<sup>a</sup>Effective number in parentheses

# EFFECT OF THE TRICHOHECENE MYCOTOXIN DIACETOXYSCIRPENOL ON NITROSAMINE-INDUCED OESOPHAGEAL CANCER AND ON RELEVANT ENZYMES IN OESOPHAGUS AND LIVER

V.M. Craddock

*MRC Toxicology Unit, Woodmansterne Road,  
Carshalton, Surrey, UK*

To test the concept that human oesophageal cancer is initiated by nitrosamines and potentiated by consumption of food contaminated by mycotoxins produced by *Fusaria*, the effect of the trichothecene mycotoxin diacetoxyscirpenol (DS), alone and in combination with *N*-nitroso-*N*-methylbenzylamine (NMBzA), on rat oesophagus was studied. Chronic treatment with DS induced oesophageal hyperplasia, but simultaneous treatment with NMBzA tended to inhibit rather than to enhance carcinogenesis. The mycotoxin did not inhibit *O*<sup>6</sup>-alkylguanine-DNA alkyltransferase in oesophagus and produced a marked induction of repair protein in liver. Depletion of the repair protein in oesophagus brought about by injection of NMBzA was not inhibited by the mycotoxin, and its reappearance was not delayed. Intubation of DS reduced DNA synthesis in the oesophagus, while dietary treatment resulted in an increase after nine weeks. The results suggest that, while simultaneous treatment with DS reduces cancer induced by NMBzA, if there is first exposure to mycotoxin and induction of hyperplasia, and then exposure to nitrosamine, so that the carcinogen acts on a vulnerable oesophagus in which there is an increased rate of cell proliferation, the mycotoxin could well enhance carcinogenesis.

Epidemiological studies have implicated nitrosamines in oesophageal cancer (Yang, 1980) and have also shown an association of the cancer with consumption of food contaminated by *Fusaria* (Marasas *et al.*, 1981), although extracts of *Fusaria* cultures have not been shown to cause cancer of the oesophagus in animal experiments (Marasas *et al.*, 1984). The concept that oesophageal cancer is initiated by nitrosamines and potentiated by trichothecene mycotoxins produced by *Fusaria* was tested by studying the effect of DS on NMBzA-induced oesophageal cancer and on enzymes likely to be involved in nitrosamine carcinogenesis.

The effect of chronic treatment with DS on oesophageal cancer in rats induced by feeding NMBzA was studied as described previously (Craddock *et al.*, 1986). *O*<sup>6</sup>-Alkylguanine-DNA alkyl transferase, the alkyl acceptor protein (AAP), was determined in extracts of oesophagus by assay of the <sup>3</sup>H-methylcysteine formed in AAP on incubation with labelled methylated DNA (Craddock & Henderson, 1986). Ornithine decarboxylase was assayed by measuring the formation of <sup>14</sup>C-carbon dioxide (Sasaki *et al.*, 1983) or of <sup>14</sup>C-putrescine (Otani *et al.*, 1984) from appropriately labelled ornithine. The method used for measuring DNA synthesis in gastric mucosa (Furihata *et al.*, 1985) was applied to oesophageal epithelium.

DS fed in the diet induced hyperplasia in the basal cells of the oesophagus, but when it was fed with NMBzA it did not increase the incidence of oesophageal papillomas or of tumours invading the submucosa or cause subthreshold doses of nitrosamine to become carcinogenic. Conversely, it tended to inhibit carcinogenesis (Table 1).

**Table 1. Effect of feeding DS, NMBzA or DS plus NMBzA on tumour incidence in rat oesophagus**

Body weight at start	Treatment	Dose (ppm)	Duration (weeks)	Incidence of papillomas <sup>a</sup>	
100 g	DS	10	10	0/9	
	NMBzA	4	10	0/5	
	NMBzA	8	10	0/5	
	NMBzA	12	10	0/5	
	NMBzA	16	10	2/5	
	DS+NMBzA	10 + 4	10	0/5	
	DS+NMBzA	10 + 8	10	0/5	
	DS+NMBzA	10 + 16	10	2/10	
	Weanlings	DS	10	10-11	0/9
		NMBzA	16	10-11	4/10
DS+NMBzA		10 + 16	10-11	0/9	
100 g	DS	7	14	0/5	
	NMBzA	16	14	2/5 (large)	
	DS+NMBzA	7 + 16	14	2/5	
	DS	7	16	0/5	
	NMBzA	16	16	4/5 (large)	
	DS+NMBzA	7 + 16	16	1/4	

<sup>a</sup>Number of animals per group with papillomas of > 1 mm

As treatment with NMBzA results in alkylation of oesophageal DNA (Hodgson *et al.*, 1980), the propensity of DS to potentiate oesophageal cancer was tested by studying its effect on the repair protein AAP. Although DS can inhibit sulphhydryl-dependent enzymes (Ueno & Matsumoto, 1975), it did not affect the activity of AAP in oesophagus and caused a marked induction of the repair protein in liver (Table 2). As several hepatotoxins induce AAP in rat liver, the result suggests that DS is hepatotoxic. It may therefore play a role in the liver cancer induced by feeding animals extracts of *Fusaria* cultures (Marasas *et al.*, 1984). The fact that DS did not inhibit depletion of AAP brought about by injection of NMBzA (Table 2) implies that it does not markedly inhibit metabolism of the nitrosamine. In spite of the fact that DS can inhibit protein synthesis, it did not delay the reappearance of AAP.

**Table 2. AAP activity in liver and oesophagus of rats after treatment with DS**

Treatment	AAP activity (fmol/mg protein)	
	Liver	Oesophagus
Control	64	
Preincubation with DS	62	
Control	71	19
DS (intraperitoneal injection)	78	20
Control	70	20
DS (intubation)	114	15
Control	75	14
DS (fed at 10 ppm for 10 weeks)	75	15
Untreated		10.6; 10.6
NMBzA; DS + NMBzA		
day 1		0.6; 0.4
day 2		0.3; 0.0
day 3		10.7; 7.7

oesophagus. Sufficient data were accumulated, however, to show that DS did not induce ornithine decarboxylase in oesophagus or in liver. The mycotoxin did not inhibit the induction of the enzyme in liver brought about by injection of *N*-nitrosodiethylamine or by phorbol ester.

A well-documented *in-vitro* effect of DS is inhibition of DNA synthesis (Ueno, 1985), and intubation of DS was shown to reduce DNA synthesis in oesophagus. It is possible that in animals treated simultaneously with NMBzA the inhibition is especially pronounced in cells already damaged by the nitrosamine, so that replication of alkylated DNA would be reduced. This could account for the inhibitory effect of DS on carcinogenesis. Dietary treatment with DS for nine to 11 weeks increased DNA synthesis, in correlation with the thickening of the epithelium seen histologically. This suggests that nitrosamine treatment at the time when there is an increase in cell proliferation could well result in a higher cancer incidence.

The experiments suggest that trichothecenes are a factor in *Fusaria* cultures responsible for the oesophageal hyperplasia demonstrated by Marasas *et al.* (1981). Hence, in the human situation, simultaneous consumption of mould-contaminated food and of nitrosamines may not result in a potentiating effect of the mycotoxin. However, if there is first exposure to the mycotoxin and consequent induction of oesophageal hyperplasia, so that

Induction of ornithine decarboxylase has been used as a test for tumour promoters, not only in skin but also in organs of the gastrointestinal tract (Takano *et al.*, 1984; Furihata *et al.*, 1985). Determination of the enzyme in oesophagus was made difficult by the erratic, very high levels of activity that were frequently found, irrespective of the method of assay. Unlike the activity in liver, these high values were not completely inhibited by  $\alpha$ -methylornithine or by difluoromethylornithine. Experiments with catalase and with desferal suggested that the erratic activity was not due to decomposition of ornithine by hydrogen peroxide or by free-radical reactions. A likely explanation was the occurrence of bacteria, which were shown by histological examination frequently to adhere firmly to the keratinous surface of the

nitrosamines consumed later would act on a more vulnerable oesophagus in which there is an increased rate of cell proliferation, the mycotoxin under these conditions could well enhance carcinogenesis.

**Acknowledgements**

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# ANALYSIS OF *N*-NITROSAMINES FOR GENOTOXICITY IN PRIMARY HEPATOCTES DERIVED FROM VARIOUS SPECIES

P. Schmezer, B.L. Pool, R. Preussmann & D. Schmähl

*Institute for Toxicology and Chemotherapy, German Cancer Research Center,  
6900 Heidelberg, Federal Republic of Germany*

DNA single-strand breaks (SSB) were induced in metabolically competent primary hepatocytes from rats, hamsters and pigs by a series of *N*-nitrosamines with organ-specific activities. Both hepatotropic and nonhepatotropic carcinogens were genotoxic in the liver cells of all three species. Nonhepatotropic compounds were active at relatively lower doses than liver carcinogens in all species tested, substantiating many previous findings that organ-specific activation is not the primary determining factor of organ susceptibility to cancer. In 11 experiments of almost identical quality, the degree of SSB induced by 6.25  $\mu\text{mol}$  *N*-nitrosodimethylamine varied by 75%. This high interindividual variability, even among hepatocytes derived from a single rat strain, indicates that the slight differences seen between rat, hamster and pig hepatocytes are not necessarily due to differences in species susceptibility.

Studies of different species are aimed at determining which model most closely reflects the biological activities of a compound in humans. In most studies so far reported, nitrosamines have been activated by subcellular liver fractions using the *Salmonella typhimurium* mutagenicity assay (Prival & Mitchell, 1981; Lijinsky & Andrews, 1983). It has been found that the hamster is more active than the rat in converting several nitrosamines to bacterial mutagens. Metabolic conversion within subcellular fractions may be highly artificial, however, as the balance of activating and inactivating enzymes is not maintained (Glatt *et al.*, 1981). A more relevant metabolizing system for use *in vitro* is provided by intact primary cells, since cultivated cells lose essential enzymes. We have therefore studied the capacities of rat, hamster and pig hepatocytes to generate genotoxic metabolites from the hepatocarcinogens *N*-nitrosodimethylamine, *N*-nitrosodiethylamine, *N*-nitrosomorpholine and *N*-nitrosodiethanolamine. We also tested a compound that induces both liver and bladder tumours in rats (*N*-nitrosodibutylamine), as well as the rat oesophageal carcinogens *N*-nitrosomethylbenzylamine and *N*-nitrosoethylvinylamine, and, in addition, *N*-nitrosodibenzylamine, which has been reported to be noncarcinogenic (Druckrey *et al.*, 1967).

Hepatocytes were isolated from rats and hamsters using the two-step perfusion technique of Bradley and Sina (1984), and pig hepatocytes according to Ballet *et al.* (1984). Genotoxicity was monitored *via* induction of DNA SSB by incubating  $2 \times 10^6$  cells with appropriate concentrations of the nitrosamines dissolved in 10  $\mu\text{l}$  dimethyl sulfoxide (DMSO) and determining the induced SSB by the alkaline elution method (Kohn *et al.*, 1981; Sina *et al.*, 1983; Frei *et al.*, 1986).

The results obtained for one representative experiment are shown in Table 1.

**Table 1. Induction of DNA SSB by organotropic *N*-nitrosamines in hepatocytes derived from three different animal species**

Compound	Concentration ( $\mu\text{mol}/\text{tube}$ )	DNA eluted (difference from controls, %)		
		Rat	Hamster	Pig
<i>N</i> -Nitrosodimethylamine	1.56	13.7	7.8	-11.6
	3.13	7.3	13.1	3.4
	6.25	11.4	16.3	6.2
	12.5	25.0	36.5	15.6
	25	-	34.3	20.8
	50	-	-	30.7
<i>N</i> -Nitrosodiethylamine	1.56	-	5.5	-
	3.13	-	8.3	-
	6.25	5.6	13.7	3.6
	12.5	11.9	20.1	-
	25	30.7	27.1	31.5
	-	-	-	-
<i>N</i> -Nitrosomorpholine	1.56	21.5	-2.1	29.1
	3.13	12.1	1.5	34.6
	6.25	20.4	3.6	36.0
	12.5	13.1	11.4	33.8
	25	23.5	26.5	39.1
	50	38.5	-	-
<i>N</i> -Nitrosodiethanolamine	3.13	-	5.9	-
	6.25	-	7.5	-
	12.5	50.8	12.6	-
	25	38.4	22.3	-
	50	56.4	32.4	-
	-	-	-	-
<i>N</i> -Nitrosodibutylamine	0.63	-	22.1	-
	1	3.7	-	-
	1.25	-	26	-
	2.5	21.6	-	-
	5	28.1	-	-
	-	-	-	-
<i>N</i> -Nitrosomethylbenzylamine	0.31	17.1	39.7	-
	0.63	23.7	45.8	-
	1.25	-	44.7	44.6
	2.5	-	61.7	53.8
	-	-	-	-
<i>N</i> -Nitrosoethylvinylamine	6.25	48.2	60.8	-
	12.5	62.4	73.9	-
	25	75.7	73.3	-
	50	81.7	88.4	-
	-	-	-	-
<i>N</i> -Nitrosodibenzylamine	0.39	-	10.4	-
	0.78	50.4	31.9	-
	1.56	51.5	-	-
	3.13	57.2	-	-
	6.25	58.0	-	-
	-	-	-	-

<sup>a</sup>-, not tested

All the *N*-nitroso compounds tested were genotoxic in the hepatocytes of each of the three species. Those carcinogens that induce tumours preferentially elsewhere than in the liver (*N*-nitrosodibutylamine, *N*-nitrosoethylvinylamine, *N*-nitrosomethylbenzylamine and *N*-nitrosodibenzylamine) exert their genotoxic effects at lower doses than do the liver carcinogens. These results are in agreement with those of many previous studies, indicating that organ- and cell-specific activation does not play a decisive role in the induction of liver tumours. In comparing the three species, *N*-nitrosodiethylamine at a dose of 25  $\mu\text{mol}/\text{tube}$  was approximately equally genotoxic in rat, hamster and pig hepatocytes. At lower concentrations, however, the hepatocytes derived from hamsters were somewhat more effective in generating genotoxic metabolites. Activation of *N*-nitrosomorpholine was greatest in pig hepatocytes, followed by hamster and then rat at high concentrations and by rat and then hamster at low concentrations. In contrast, *N*-nitrosodimethylamine appears to be more genotoxic in hamster hepatocytes than in those of rat or pig.

Differences in the induction of SSB may also be due to the differential quality of the cell preparations, as well as to differences in the technical performance of individual experiments. Cell suspensions with high viability have a relatively low incidence of spontaneous SSB (expressed as % DNA retained on filter in DMSO control). Therefore, we employ cell preparations with at least 80% viability after preparation, and evaluate only those concentrations of compounds which are not toxic (> 70% viability, based on survival in DMSO control = 100%). Additionally, only high (preferably > 70%) values of '% DNA retained on filters in control' reflect adequate technical performance of individual experiments. On the basis of different levels of spontaneous SSB, interindividual variation in a series of assay parameters are shown in Table 2. It is apparent that, even in those experiments of almost identical quality (group III), deviations in viability after isolation and after incubation with DMSO or *N*-nitrosodimethylamine, as well as in the values '% DNA retained on filter in controls' are relatively small in comparison to the great differences observed for the induction of SSB by *N*-nitrosodimethylamine (63-75%). This surprisingly high variability of individual hepatocyte preparations obtained from a single strain of rats obviates all effects that may be interpreted as species differences.

**Table 2. Interindividual variability of various test parameters in repeated experiments to determine induction of DNA SSB by *N*-nitrosodimethylamine (NDMA) in rat hepatocytes**

Group <sup>a</sup>	No. <sup>b</sup>	Hepatocytes, % viable			Incidence of SSB		
		After isolation	DMSO <sup>c</sup> (1 h)	NDMA <sup>d</sup> (1 h)	DMSO control <sup>e</sup>	NDMA test <sup>f</sup>	
I	Mean $\pm$ SD	19	89.9 $\pm$ 3.6	67.2 $\pm$ 10.8	93.4 $\pm$ 17	68.2 $\pm$ 18	29.1 $\pm$ 18.3
	range		(81.5-94.8)	(37-84)	(70-144)	(27.4-91.8)	(0-69.4)
	% deviation		4	16	18	26	63
II	Mean $\pm$ SD	16	89.9 $\pm$ 3.8	68.4 $\pm$ 17.9	94.6 $\pm$ 17.9	74.8 $\pm$ 9.8	30.5 $\pm$ 19.2
	range		(81.5-94.8)	(37-84)	(70-144)	(56-91.8)	(0-69.4)
	% deviation		4	17	19	13	63
III	Mean $\pm$ SD	11	90.7 $\pm$ 3.5	67.1 $\pm$ 13.7	95.8 $\pm$ 19.5	79.1 $\pm$ 7.9	28.1 $\pm$ 21.2
	range		(81.5-94.8)	(37-84)	(75-144)	(70.5-91.8)	(0-69.4)
	% deviation		4	20	20	10	75

<sup>a</sup>Minimum '% DNA retained on filters in the DMSO controls' are lowest for Group I (> 30%, all experiments performed) followed by Group II (> 50%, for 16 or 19 experiments) and highest for Group III (> 70%, 11 experiments)

<sup>b</sup>n, number of experiments included in calculations

<sup>c</sup>Absolute proportion of viable cells after 1-h incubation with 10  $\mu\text{l}$  DMSO at 37°C in shaking water bath

<sup>d</sup>6.25  $\mu\text{mol}/\text{tube}$ ; viability in test was based on 100% survival in DMSO controls

<sup>e</sup>Absolute value '% DNA retained on filters'

<sup>f</sup>6.25  $\mu\text{mol}/\text{tube}$ ; values are the % increase of DNA elution over controls (see Table 1)



In conclusion, the pronounced species variation observed in the *S. typhimurium*/-microsomal assay, in which mainly the extent of metabolic activation is measured, was not seen here. Activation to bacterial mutagens may be due primarily to cytochrome P450 enzymes, and the extent of this activation is determined by the levels of enzymes present and by their activity, which in turn is related to the quality and quantity of added cofactors. In contrast, the effects in the hepatocyte system reflect metabolic activation and deactivation by all available enzymes, many of which have not yet been identified. The net yield of genotoxic effects may also be enhanced by toxicity or diminished by DNA repair. Furthermore, toxicity, genotoxicity and repair are greatly dependent on the type of DNA insult (e.g., alkylation *versus* arylation). Thus, species variability, which is relatively wide with regard to subcellular metabolic activation, as indicated by specific mutations in repair-deficient bacteria, may be hidden by high interindividual variability, which is due to the additional factors in intact primary mammalian cells, as discussed above. Therefore, this assay is suitable only for qualitative determinations of the specific genotoxic activity of a compound. For quantitative evaluations, either a large number of experiments with cells from individual animals must be performed or results should be evaluated from duplicated experiments using pooled hepatocytes of different animals.

**INDUCTION OF DNA REPAIR IN HeLa S3 CARCINOMA  
CELLS BY THE N-NITROSO DERIVATIVES OF  
1-(N-L-TRYPTOPHAN)-1-DEOXY-D-FRUCTOSE  
AND 1-(5-HYDROXYTRYPTAMINO)-1-DEOXY-D-FRUCTOSE**

S.C. Lynch, P.J. Eckert & D.W. Gruenwedel

*Department of Food Science and Technology,  
University of California, Davis, CA 95616, USA*

**HeLa S3 cells, when incubated at 37°C with the *N*-nitroso derivative of the Amadori compounds 1-(*N*-L-tryptophan)-1-deoxy-D-fructose (FRU-TRP) or 1-(5-hydroxytryptamino)-1-deoxy-D-fructose (FRU-SEROT) in the presence of a six-fold molar excess of sodium nitrite, exhibit increased intracellular DNA synthesis. Sodium nitrite alone, at identical levels, elicits a similar response, albeit to a much lesser degree. No response whatsoever is produced when the cells are incubated with the parent Amadori compounds. The observed stimulation of DNA replication is DNA repair. Two major routes are suggested by which nitrosated FRU-TRP (NO-FRU-TRP) and nitrosated FRU-SEROT (NO-FRU-SEROT) could damage intracellular DNA.**

As reported by Coughlin and collaborators (Coughlin, 1979; Russell, 1983), the *N*-nitroso derivative of the Amadori compound NO-FRU-TRP, but not the parent compound, FRU-TRP, displays considerable mutagenic activity in the Ames test. This has been confirmed by Röper *et al.* (1984), also using the Ames test, and by us (Lynch *et al.*, 1983; Gruenwedel *et al.*, 1984), utilizing HeLa S3 carcinoma cells and a number of macromolecular synthetic events (i.e., DNA, RNA and protein synthesis) as metabolizing system and mutagenesis endpoints, respectively. The present communication expands on the previous work by reporting also on the effects of the Amadori compound FRU-SEROT and its *N*-nitroso derivative, NO-FRU-SEROT, on intracellular DNA synthesis in HeLa S3 cells.

As can be seen from Table 1, neither FRU-TRP nor FRU-SEROT (the latter prepared according to the method of Mester and Mester, 1975) had any marked effect on DNA replication in HeLa S3 suspension-culture cells, whether they were exposed for extended periods of time at a given substance concentration (upper part of the table) or to varied substance concentrations at given incubation periods (lower part of the table). The *N*-nitroso derivatives NO-FRU-TRP and NO-FRU-SEROT, however, increased DNA replication dramatically, exceeding by far the response elicited by sodium nitrite alone.

Since none of the compounds affects cell viability (identical with that of the control) or RNA and protein synthesis (also identical with those of the control), stimulation of macromolecular synthesis appears to be DNA-specific. This assumption was verified by incubating the cells under conditions (Lynch *et al.*, 1983) that do not permit normal replication but only repair synthesis ('unscheduled DNA synthesis', UDS). It was found that

**Table 1. Effects of FRU-TRP, FRU-SEROT, NO-FRU-TRP and NO-FRU-SEROT and of sodium nitrite on DNA replication as a function of incubation time at given substance concentrations, and of substance concentration at given incubation periods**

Incubation time <sup>a,b</sup>					
Time (h)	[FRU-TRP] DNA	[FRU-SEROT] DNA	[NO-FRU-TRP] DNA	[NO-FRU-SEROT] DNA	[NaNO <sub>2</sub> ] DNA
1	92	100	73	80	109
3	111	105	80	100	100
6	108	99	80	156	104
12	99	95	160	400	140
24	111	100	251	200	146
36	94	-	180	-	140

Substance concentration (molar) <sup>b,c</sup>					
-Log dose	[FRU-TRP] DNA	[FRU-SEROT] DNA	[NO-FRU-TRP] DNA	[NO-FRU-SEROT] DNA	[NaNO <sub>2</sub> ] DNA
7.00	95	100	114	100	-
6.50	89	-	125	-	-
6.22	-	-	-	-	83
6.00	86	99	161	95	-
5.74	-	-	-	-	82
5.50	100	85	151	110	-
5.22	-	-	-	-	95
5.00	107	90	160	120	-
4.74	-	-	-	-	85
4.50	80	110	179	150	-
4.22	-	-	-	-	88
4.00	98	125	188	400	-
3.74	-	-	-	-	101
3.50	114	-	212	-	-
3.22	-	-	-	-	132
3.00	111	-	251	-	-
2.74	-	-	-	-	146

<sup>a</sup>At given concentrations of: FRU-TRP, 1 mM; FRU-SEROT, 0.1 mM; NO-FRU-TRP, 1 mM (in presence of 6 mM NaNO<sub>2</sub>); NO-FRU-SEROT, 0.1 mM (in presence of 0.6 mM NaNO<sub>2</sub>); NaNO<sub>2</sub>, 6 mM

<sup>b</sup>The numbers under the heading DNA are % values of label incorporation (labelling of the control = 100%). [methyl-<sup>3</sup>H]Thymidine served as the precursor of DNA synthesis.

<sup>c</sup>At given incubation periods of: 24 h for FRU-TRP, NO-FRU-TRP and NaNO<sub>2</sub>, and 12 h for FRU-SEROT and NO-FRU-SEROT

the 'stimulation' noted in Table 1 is indeed DNA repair: for instance, 0.1 mM NO-FRU-SEROT, in the presence of 0.6 mM sodium nitrite, causes after 24 h of incubation a 3.2-fold increase in UDS, even over that caused by 0.6 mM sodium nitrite alone within the same incubation period. Needless to say, FRU-SEROT alone does not induce DNA repair. This then is in total agreement with what has been shown by us to hold for FRU-TRP and NO-FRU-TRP (Lynch *et al.*, 1983; Gruenwedel *et al.*, 1984).

One of two possible causes of the DNA damage could be operative. (1) As typical *N*-nitrosamines, even activated at the  $\beta$ -positions, the compounds could decompose, following the route of  $\alpha$ -,  $\beta$ - or  $\omega$ -oxidation, to yield alkyl or arylalkyl carbonium ions that interact with a number of nucleotide binding sites of importance to intrastrand DNA hydrogen bonding. Disruption of Watson-Crick hydrogen bonding would then be the damaging event. (2) The two Amadori compounds, mimicking nucleotides structurally, intercalate with DNA. While this may not represent damage *per se*, intercalation of the *N*-nitroso compounds may result in tryptophan-DNA and tryptamine-DNA adduct formation, not unlike the photoinduced tryptophan-pyrimidine adduct formation known to occur in DNA (see Reeve & Hopkins, 1980). Distortion of base stacking would then be the damaging event. Research utilizing circular dichroism spectroscopy has started in our laboratory to investigate possibility (2).

The public health implications of the mutagenic properties of the *N*-nitroso derivatives of the two Amadori compounds are unknown at present. However, in view of the readiness with which amino acids in general react with aldoses upon heating to yield Amadori compounds, and in view of the ease with which these compounds are converted completely to their *N*-nitroso derivatives when incubated with sodium nitrite under mildly acidic conditions at temperatures of physiological interest (Coughlin, 1979; Heyns *et al.*, 1979), the question of the health risks posed by this conversion is of considerable importance. Currently, we are carrying out a systematic study of a large variety of *N*-nitrosated Amadori compounds for their ability to induce UDS in HeLa S3 cells and to cause phenotypic changes such as 'cell transformation' in suitable mammalian cells.

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# SYNTHESIS, ANALYSIS AND MUTAGENIC ACTIVITY OF *N*-NITROSO DERIVATIVES OF GLYCOSYLAMINES AND AMADORI COMPOUNDS: NITROSATED MODEL SUBSTANCES FOR THE EARLY MAILLARD REACTION PRODUCTS

B. Pignatelli<sup>1</sup>, C. Malaveille<sup>1</sup>, M. Friesen<sup>1</sup>, A. Hautefeuille<sup>1</sup>, H. Bartsch<sup>1</sup>,  
D. Piskorska<sup>2</sup> & G. Descotes<sup>2</sup>

<sup>1</sup>International Agency for Research on Cancer, 150 cours Albert-Thomas,  
Lyon, France; and <sup>2</sup>Laboratory of Organic Chemistry U463,  
CNRS-PIREN, University of Lyon I, 43 Bd du 11 Novembre 1918,  
69622 Villeurbanne, France

A series of nine glycosylamines and an Amadori compound and their *N*-nitroso derivatives were synthesized. The structures were ascertained by spectroscopy and elemental analysis. The *N*-nitroso compounds were further characterized by denitrosation with hydrogen bromide-acetic acid, followed by detection of the liberated NO by a chemiluminescence detector. *N*-Nitroso derivatives of *N*-*p*-nitrophenyl/*p*-methylphenyl/*p*-carboxyphenyl pentosylamines, *N*-*p*-methylphenyl-1-deoxy-D-fructosylamine (Amadori compound) and *N*-3-ethylindole-D-xylosylamine were shown to be directly-acting mutagens in *Salmonella typhimurium* TA100. The activity of some of the compounds was similar to that of *N*-ethyl-*N*-nitrosourea. Their mutagenic activity was shown to be dependent on the structure of the amine and the sugar moieties and requires the presence of free hydroxyl groups in the sugar. The mutagenicity of *N*-nitrosoglycosylamines was attributed to their hydrolysis to arene diazonium cations. Their formation was detected *via* azo-coupling with *N*-ethyl-1-naphthylamine, using spectrophotometric and mass-spectrometric analyses. Our data implicate arene (alkyl) diazonium cations as the ultimate mutagens of *N*-nitrosoglycosylamines and *N*-nitroso Amadori compounds, a little explored class of *N*-nitroso compounds which may be formed *in vivo*.

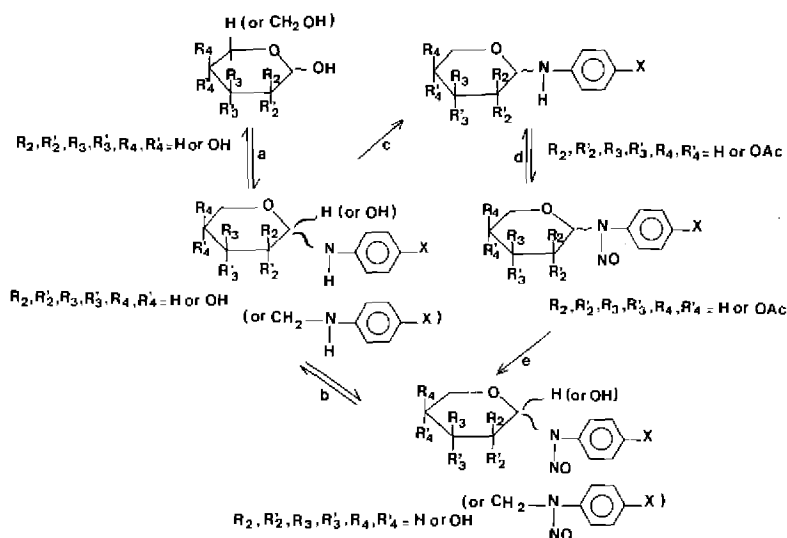
Glycosylamines and Amadori compounds are present in many food items and are formed during the early stages of nonenzymatic browning (Maillard) reactions (Hodge, 1953; Ericksson, 1981). The secondary amino group present in these compounds can be nitrosated. Of the synthetic *N*-nitroso-fructose-amino acids, only derivatives of tryptophan, histidine and threonine have been reported to be directly-acting mutagens in *S. typhimurium his<sup>-</sup>* strains, although the reaction mechanism has not been described (Coughlin 1979; Röper *et al.*, 1982; Pool *et al.*, 1984; Röper *et al.*, 1984).

The aims of the study were to elucidate the structural parameters that determine the mutagenic potency of *N*-nitrosoglycosylamines and *N*-nitroso Amadori compounds and to investigate the nature of the ultimate mutagen(s) derived from the parent compound. Since they are model compounds for early Maillard reaction products that may occur in food, reaction products between amines such as *p*-toluidine, *p*-aminobenzoic acid, *p*-nitroaniline and tryptamine, and sugars such as pentoses and fructose and their *N*-nitrosated derivatives were synthesized, characterized and tested for their mutagenicity in *S. typhimurium* TA100.

### Synthesis, spectroscopic characterization and analysis of *N*-glycosylamines and their *N*-nitrosated derivatives

Compounds listed in Table 1 were synthesized according to sequential reactions schematized in Figure 1. All of the glycosylamines and the Amadori compound were nitrosated by nitrite, except T-Xyl which was nitrosated by nitrogen tetroxide (Tsujiyama *et al.*, 1981). The purity of the *N*-nitroso compounds was verified by thin-layer chromatography, giving single spots with a positive response to Griess reagent. The principal absorption bands/maxima of the infra-red/ultra-violet spectra confirmed the presence of the major functional groups/transition states of the synthetic compounds.

**Fig. 1.** Synthesis of *N*-nitroso-1-deoxyfructosylamine and *N*-nitroso D- or L-pentopyranosylamines



(a) substituted phenylamines; (b)  $NO_2^-$ ,  $HCl$ ; (c)  $Ac_2O$ , pyridine; (d)  $NO_2^-$ ,  $AcOH$ ; (e)  $CH_3O^-$ ,  $CH_3OH$ . For technical details see Pignatelli *et al.* (1986).

The proton nuclear magnetic resonance spectra of the *O*-acetylated pentosylamines and their *N*-nitroso derivatives clearly showed the electron withdrawing effect of the nitroso group, which deshielded all the protons.

Using the method of Walters *et al.* (1983), the *N*-NO content of acetylated and nonacetylated *N*-nitroso derivatives varied from 84-100% and 60-93%, respectively (data not shown and Table 1). These results were in agreement with those of the elemental analyses (data not shown). High-performance liquid chromatography (HPLC) analysis of *N*(NO)-CP-Ara, for which the lowest purity was obtained, revealed only the corresponding glycosylamine as an impurity.

Table 1. Glycosylamines/Amadori compounds and their N-nitroso derivatives tested for mutagenic activity

Structures	Substituents		Glycosylamine/Amadori compound and N-nitroso derivative	TEA <sup>a</sup> analysis of N-NO content: purity (%)	Abbreviation
	X	Y			
	NO <sub>2</sub>	H	N- <i>p</i> -nitrophenyl-D-ribose/amine	-	NP-Rib
	NO <sub>2</sub>	NO	N-nitroso-N- <i>p</i> -nitrophenyl-D-ribose/amine	65	N(NO)-NP-Rib
	ND <sub>2</sub>	H	N- <i>p</i> -nitrophenyl-L-arabino/amine	-	NP-Ara
	NO <sub>2</sub>	NO	N-nitroso-N- <i>p</i> -nitrophenyl-L-arabino/amine	93	N(NO)-NP-Ara
	CH <sub>3</sub>	H	N- <i>p</i> -methylphenyl-D-arabino/amine	-	MP-Ara
	CH <sub>3</sub>	NO	N-nitroso-N- <i>p</i> -methylphenyl-D-arabino/amine	70	N(NO)-MP-Ara
	CDOH	H	N- <i>p</i> -carboxyphenyl-D-arabino/amine	-	CP-Ara
	COOH	NO	N-nitroso-N- <i>p</i> -carboxyphenyl-D-arabino/amine	60	N(NO)-CP-Ara
	NO <sub>2</sub>	H	N- <i>p</i> -nitrophenyl-D-xylo/amine	-	NP-Xyl
	ND <sub>2</sub>	NO	N-nitroso-N- <i>p</i> -nitrophenyl-D-xylo/amine	90	N(NO)-NP-Xyl
	CH <sub>3</sub>	H	N- <i>p</i> -methylphenyl-D-xylo/amine	-	MP-Xyl
	CH <sub>3</sub>	NO	N-nitroso-N- <i>p</i> -methylphenyl-D-xylo/amine	87	N(NO)-MP-Xyl
	COOH	H	N- <i>p</i> -carboxyphenyl-D-xylo/amine	-	CP-Xyl
	COOH	NO	N-nitroso-N- <i>p</i> -carboxyphenyl-D-xylo/amine	72	N(NO)-CP-Xyl
	NO <sub>2</sub>	H	N- <i>p</i> -nitrophenyl-D-lyxo/amine	-	NP-Lyx
	NO <sub>2</sub>	ND	N-nitroso-N- <i>p</i> -nitrophenyl-D-lyxo/amine	69	N(NO)-NP-Lyx
		H	N-3-ethylindole-D-xylo/amine	-	T-Xyl
		ND	N-nitroso-N-3-ethylindole-D-xylo/amine	99	N(NO)-T-Xyl
	CH <sub>3</sub>	H	N- <i>p</i> -methylphenyl-1-deoxy-D-fructosylamine	-	MP-Fru
	CH <sub>3</sub>	ND	N-nitroso-N- <i>p</i> -methylphenyl-1-deoxy-D-fructosylamine	84	N(NO)-MP-Fru

<sup>a</sup> Thermal energy analysis

### Structure-mutagenic activity relationships

Three out of nine glycosylamines (MP-Ara, MP-Xyl, T-Xyl) were found to be weakly mutagenic in *S. typhimurium* TA100 strain (Table 2); all the other glycosylamines and the Amadori compound were devoid of mutagenic activity when tested at up to 4 mg/assay (Table 2 and data not shown). The *N*-nitrosation of seven out of ten of these compounds converted them into directly-acting mutagens (Tables 2 and 3). With the three weakly mutagenic glycosylamines, *N*-nitrosation yielded compounds that had either higher (T-Xyl), unchanged (MP-Ara) or decreased (MP-Xyl) mutagenicity.

**Table 2. Effect of *N*-nitrosation on the mutagenicity of glycosylamines and an Amadori compound in *Salmonella typhimurium* TA100**

Compound (conc. range tested in mg/assay)	Mutagenicity (rev./mg) <sup>a</sup>	
	Before nitrosation	After nitrosation
CP-Xyl <sup>b</sup> (0.5-2)	0	35
CP-Ara <sup>b</sup> (0.3-3)	0	180
MP-Fru <sup>b</sup> (0.3-4)	0	76
MP-Ara <sup>b</sup> (0.25-4)	98	98
MP-Xyl <sup>b</sup> (0.25-2)	32	< 7
T-Xyl <sup>c</sup> (0.016-1)	<75	21895

<sup>a</sup>The specific mutagenicities were calculated from the pseudo-linear part of dose-response curves obtained from 2-3 series of duplicate experiments.

<sup>b</sup>Tested using liquid incubation assay: the test compound, dissolved in 200  $\mu$ l ethanol:0.9% NaCl in 5 mM Sørensen phosphate buffer, pH 7.4 (20:80; v:v) and  $9-18 \times 10^8$  bacteria (300  $\mu$ l concentrated culture medium) were mixed with 300  $\mu$ l 68 mM Sørensen phosphate buffer, pH 7.4 and incubated at 37°C with shaking for 3 h then plated and processed as described previously (Malaveille *et al.*, 1982). For further details, see Pignatelli *et al.* (1986).

<sup>c</sup>Tested using liquid incubation assay: the test compound, dissolved in 200  $\mu$ l 68 mM Sørensen phosphate buffer, pH 7.4, and  $3-6 \times 10^8$  bacteria (100  $\mu$ l concentrated culture medium) were mixed, incubated for 3 h at 37°C and plated.

### Reaction mechanism by which *N*-nitroso derivatives of glycosylamines and the Amadori compound exert their mutagenicity

*N*-Nitrosated glycosyl-*p*-nitroaniline has been shown to decompose to arenediazonium cations through nonenzymatic hydrolysis (Bognar, 1973; Fig. 2). Experiments were therefore carried out to examine whether the mutagenicity of *N*-nitrosoglycosylamines and of the *N*-nitroso Amadori compound is related to their hydrolytic decomposition into arenediazonium cations. Both *p*-methylphenyldiazonium and *p*-nitrophenyldiazonium cations (tested as fluoborate salts), which should be derived from *N*(NO)-MP-Fru and *N*(NO)-NP-Ara, respectively, were found to be directly-acting mutagens in *S. typhimurium*

A comparison of the mutagenicities in *S. typhimurium* TA100 of a series of *N*-nitrosated glycosyl-*p*-nitroanilines showed that their activity is dependent on the structure of the sugar moiety. *N*(NO)-NP-Ara, the most active mutagen in this series, is  $\approx 18$ -fold more mutagenic than *N*(NO)-NP-Rib (Table 3). The importance of the sugar moiety in determining the mutagenic activity of *N*-nitrosated glycosylamines is supported by the fact that acetylation of sugar hydroxyl groups almost completely suppressed mutagenicity (data not shown). In addition, the mutagenicity of *N*-nitrosoglycosylamines was found to be heavily dependent on the structure of the amino moiety: after 30 min of liquid incubation under the same assay conditions, the mutagenicity (expressed as revertant/ $\mu$ mol) of *N*(NO)-T-Xyl was 45-fold that of *N*(NO)-NP-Xyl (time-response curves not shown).



**Table 3. Influence of the glycosidic moiety on the mutagenicity in *S. typhimurium* TA100 of *N*-nitrosated glycosyl-*p*-nitroanilines**

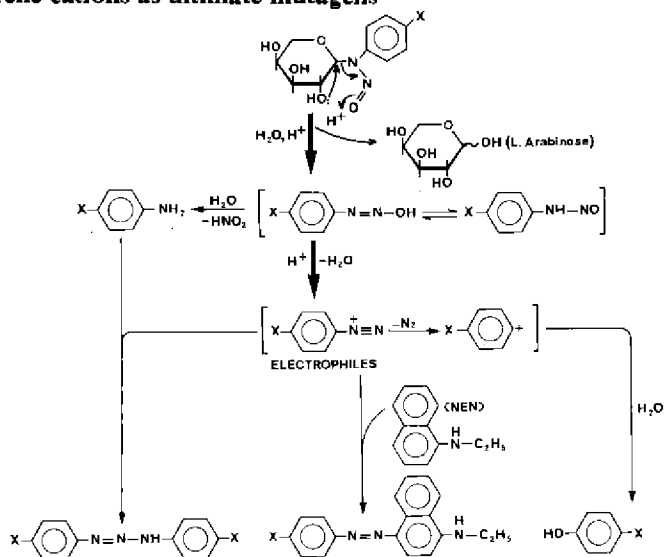
<i>N</i> -Nitrosoglycosylamine (conc. range tested in mg/assay)	Mutagenicity (rev./mg) <sup>a</sup>	Relative mutagenicity <sup>b</sup>
<i>N</i> (NO)-NP-Rib (0.5-4)	260	1
<i>N</i> (NO)-NP-Xyl (0.5-4)	570	2
<i>N</i> (NO)-NP-Lyx (0.063-2)	2770	10.5
<i>N</i> (NO)-NP-Ara (0.031-1)	4550	17.5

<sup>a</sup>The specific mutagenicities were calculated from the pseudo-linear part of dose-response curves obtained from 2-3 series of duplicate experiments. Experiments were carried out using the plate incorporation assay: the test compound, dissolved in 200 μl ethanol and 3-6 × 10<sup>8</sup> bacteria (100 μl concentrated culture medium) were plated in duplicate.

<sup>b</sup>Relative specific mutagenicity, taking the activity of *N*(NO)-NP-Rib as 1

acteria to remove unreacted chemicals and plating (Malaveille *et al.*, 1982). The data obtained have permitted a comparison of mutagenicities based on the linear part of time-response curves. After 30 min of liquid incubation, the mutagenic activity (expressed as revertants/μmol) of *p*-nitrophenyldiazonium fluoborate was 11-fold and 141-fold higher than that of *N*(NO)-NP-Ara and *N*(NO)-NP-Xyl, respectively. Thus, support for the involvement of arenediazonium cations as proximate or ultimate carcinogens was obtained.

**Fig. 2. Scheme for the hydrolysis of *N*-nitrosoglycosylamines to yield arenediazonium cations and arene cations as ultimate mutagens**



The reactions of arenediazonium cations with *N*-ethyl-1-naphthylamine (NEN), which yield the corresponding diazo derivative, and possible side reactions are also shown.

TA100 using the plate incorporation assay. When assayed under experimental conditions identical to those used to test the two phenyldiazonium salts, *N*(NO)-MP-Fru was ≈170-fold less and *N*(NO)-NP-Ara ≈1.3-fold more mutagenic than their corresponding phenyldiazonium salts (Table 4).

To better compare the mutagenicities of *N*-nitrosoglycosylamines with that of their related phenyldiazonium derivatives, the activities of *N*(NO)-NP-Ara and *N*(NO)-NP-Xyl and *p*-nitrophenyldiazonium fluoborate were measured as a function of time of liquid incubation (up to 90 min) before centrifugation of the bac-

**Table 4. Mutagenicity of phenyldiazonium fluoborate salts and of structurally related *N*-nitroso derivatives of glycosylamines and an Amadori compound in *S. typhimurium* TA100**

Compound	Conc. range tested in $\mu\text{g}/\text{assay}$	Mutagenicity <sup>a</sup> (rev./ $\mu\text{mol}$ )
<i>p</i> -Methylphenyldiazonium fluoborate	5-80	1350
<i>N</i> (NO)-MP-Fru	1000-4000	8
<i>p</i> -Nitrophenyldiazonium fluoborate	5-80	935
<i>N</i> (NO)-NP-Ara	31-125	1175

<sup>a</sup>The specific mutagenicities were calculated from the pseudo-linear part of dose-response curves obtained from 1-3 series of duplicate experiments. Experiments were carried out using the plate incorporation assay; test compound, dissolved in 200  $\mu\text{l}$  sterile deionized water (*N*-nitrosated glycosylamine) or 40  $\mu\text{l}$  anhydrous dimethyl sulfoxide (phenyldiazonium fluoborate salts) and  $3-6 \times 10^8$  bacteria (100  $\mu\text{l}$  concentrated culture medium) were plated in duplicate. (The final volume before plating was adjusted with dimethyl sulfoxide or sterile deionized water, for test compounds dissolved in water or dimethyl sulfoxide, respectively.)

ethyl-1-naphthylamine, derived from the corresponding *N*-nitrosoglycosylamines and the *N*-nitroso Amadori compound, were identified on the basis of a comparison of ultraviolet/visible absorption spectra and of GC/mass spectra with those of authentic synthetic compounds.

At pH 3.5 (5 h incubation at 37°C), the yield of *p*-nitrophenyldiazonium cation formed from both *N*(NO)-NP-Ara and *N*(NO)-NP-Xyl was  $\approx$  65-fold higher than that of *p*-methylphenyldiazonium cation derived from *N*(NO)-MP-Fru. At pH 4.6, *N*(NO)-NP-Ara yielded twice as many *p*-nitrophenyldiazonium cations as did *N*(NO)-NP-Xyl. The acetylation of hydroxyl groups in the sugar moiety of *N*(NO)-NP-Ara suppressed the formation of *p*-nitrophenyldiazonium cations, due perhaps to the fact that the acetoxy group at the C-2 position reduced hydrolytic cleavage of the anomeric bond (Fig. 2) because of protonation of the carboxyl group and of steric hindrance.

Since (i) the specific mutagenicity of the arenediazonium cations was found to be higher than that of the corresponding *N*-nitrosoglycosylamines and the *N*-nitroso Amadori compound; (ii) the rate of formation (yield at pH 3.5 or 4.6) of arenediazonium cations from *N*-nitrosoglycosylamines paralleled their mutagenic activity (Tables 2 and 3 and data in the text); and (iii) acetylation of hydroxyl groups in the sugar moiety suppressed the mutagenicity and formation of diazonium cations, we conclude that the mutagenicity of *N*-nitrosoglycosylamines and of the *N*-nitroso Amadori compound is attributable mainly to their hydrolytic decomposition into arene (alkyl) diazonium, according to the mechanism proposed (Fig. 2).

To further ascertain the involvement of arenediazonium cations in the mutagenicity of *N*-nitrosoglycosylamines and an *N*-nitroso Amadori compound, we measured spectrophotometrically their formation by azo-coupling to *N*-ethyl-1-naphthylamine at pH ranging from 3.5 to 6.1 (Table 5). 4-(*p*-Nitrophenylazo)-*N*-ethyl-1-naphthylamine and 4-(*p*-methylphenylazo)-*N*-ethyl-1-naphthylamine were synthesized from diazonium fluoborate salts to establish calibration curves. Gas chromatography-electron impact mass spectrometry (GC-MS) was used to ascertain their structures and check their purity. 4-(*p*-Nitrophenylazo)-*N*-ethyl-1-naphthylamine and 4-(*p*-methylphenylazo)-*N*-

**Table 5. Hydrolysis of *N*-nitrosoglycosylamines and a *N*-nitroso Amadori compound into phenyldiazonium cations at various pHs**

<i>N</i> -Nitrosoglycosylamine and <i>N</i> -nitroso Amadori compound	pH	Yield of respective phenyldiazonium cation of parent compound (%) <sup>a</sup>
<i>N</i> (NO)-NP-Xyl	3.5	100
<i>N</i> (NO)-NP-Ara	3.5	100
Acetylated <i>N</i> (NO)-NP-Ara	3.5	0.5 <sup>b</sup>
<i>N</i> (NO)-MP-Fru	3.5	1.5
<i>N</i> (NO)-NP-Xyl	4.6	2.3
<i>N</i> (NO)-NP-Ara	4.6	4
	5.6	0.25
	6.1	0.06

<sup>a</sup>Formation of phenyldiazonium cations was measured by azo coupling with *N*-ethyl-1-naphthylamine after incubation at 37°C for 5 h.

<sup>b</sup>Incubation at 37°C for 5 h and at 90°C for 3 h

Humans are exposed to dietary *N*-nitrosoglycosylamines and *N*-nitroso Amadori compounds or to their precursors, which may undergo endogenous nitrosation; the latter reaction has now been shown definitely to occur in humans (Ohshima & Bartsch, 1981). It is thus important to gain more knowledge about the chemical and biological properties of this relatively unexplored class of *N*-nitroso compounds, particularly since the mutagenic potency of *N*(NO)-T-Xyl and *N*(NO)-NP-Ara (expressed as revertants in *S. typhimurium* TA100/mM concentration per min in liquid incubation) is close to that of *N*-ethyl-*N*-nitrosourea (Bartsch *et al.*, 1983b), a versatile carcinogen in many animal species.

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## **ENDOGENOUS FORMATION**

## RECENTLY IDENTIFIED NITRITE-REACTIVE COMPOUNDS IN FOOD: OCCURRENCE AND BIOLOGICAL PROPERTIES OF THE NITROSATED PRODUCTS

K. Wakabayashi, M. Nagao, M. Ochiai, Y. Fujita, T. Tahira,  
M. Nakayasu, H. Ohgaki, S. Takayama & T. Sugimura

*National Cancer Center Research Institute,  
1-1 Tsukiji 5-chome, Chuo-ku,  
Tokyo 104, Japan*

Various Japanese foodstuffs are directly-acting mutagens in *Salmonella typhimurium* TA100 after nitrite treatment. Such mutagen precursors include tyramine and  $\beta$ -carboline derivatives, isolated from soya sauce, and indole-3-acetonitrile, 4-methoxyindole-3-acetonitrile and 4-methoxyindole-3-aldehyde, isolated from fresh Chinese cabbage. A mutagen produced from tyramine with nitrite was found to be 4-(2-aminoethyl)-6-diazo-2,4-cyclohexadienone (3-diazotyramine), and one produced from indole-3-acetonitrile with nitrite to be 1-nitrosoindole-3-acetonitrile. These two mutagens were directly-acting mutagens not only in *S. typhimurium* TA100 and TA98 but also in Chinese hamster lung cells, using diphtheria toxin resistance as a selective marker. The carcinogenicity of 3-diazotyramine was demonstrated in male Fischer 344 rats. Tyramine,  $\beta$ -carboline and indole compounds are present ubiquitously in our environment, especially in foods. Therefore, the role of these newly identified mutagen precursors in the development of human cancer should be taken into consideration.

Mortality from gastric cancer is much higher in Japan than in Europe or the USA (Hirayama, 1979). A good correlation has been found between nitrate intake *per capita* and mortality from gastric cancer in various countries (Fine *et al.*, 1982). Therefore, attention has been paid to the presence of nitrosatable precursors, which could be causes of gastric cancer, in Japanese foods. We recently surveyed the mutagenicities of normal Japanese foods after nitrite treatment, and found that various foodstuffs contained mutagen precursors that were directly-acting mutagens in *S. typhimurium* TA100 after nitrite treatment. Six compounds were isolated as nitrosatable mutagen precursors. Interestingly, all these compounds differed from previously known alkylamides, and their mutagen-precursor activities were a new finding. Furthermore, one mutagen produced from a mutagen precursor after nitrite treatment was carcinogenic in rats. These experiments and the results obtained in our laboratory are described below.

### **Mutagenicities of various Japanese foodstuffs after nitrite treatment**

Soya bean fermentation products — soya sauce and bean paste — were mutagenic after treatment with 50 mM sodium nitrite at pH 3.0 for 1 h at 37°C in the dark, but soya beans were not mutagenic under the same conditions (Wakabayashi *et al.*, 1983). Among eight kinds of soya sauce produced in Japan, seven showed marked mutagenicity, inducing 9600-25 200 revertants/ml; the other soya sauce induced 2700 revertants/ml. A brand of bean paste induced 6200 revertants/g after nitrite treatment.

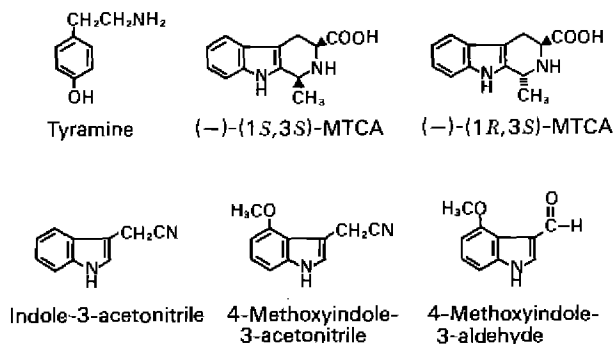
Vegetables and pickled vegetables were also directly-acting mutagens in TA100 after nitrite treatment (Wakabayashi *et al.*, 1985a, b). Fresh vegetables, such as Chinese cabbage, cabbage, radish root and spinach, induced 2400-4700 revertants/g after nitrite treatment. Eleven vegetables pickled in bean paste, rice bran, lees, soya sauce or with salt were mutagenic after nitrite treatment, inducing 1900-18 000 revertants/g. We also examined changes in precursor activity during salt fermentation of Chinese cabbage: the precursor activity of fresh Chinese cabbage decreased by 35% during fermentation for one day and then remained constant for up to ten days. The mutagen precursors in Chinese cabbage pickled with salt were therefore suggested to be derived from those in fresh Chinese cabbage, and not to be newly formed during salt fermentation. This may also be the case with other pickled vegetables.

Sun-dried herring and sardine showed mutagenicity after nitrite treatment, giving 5500 and 2400 revertants/g, respectively (Wakabayashi *et al.*, 1985a)

### Isolation and identification of nitrosatable mutagen precursors

We next tried to isolate nitrosatable mutagen precursors from soya sauce and fresh Chinese cabbage, large amounts of which are consumed in Japan. Three mutagen precursors were isolated from soya sauce by various column chromatographies and identified as tyramine (Ochiai *et al.*, 1984) and (-)-(1*S*, 3*S*)-1-methyl-1,2,3,4-tetrahydro- $\beta$ -carboline-3-carboxylic acid [(-)-(1*S*, 3*S*)-MTCA] and its stereoisomer (-)-(1*R*, 3*S*)-MTCA (Wakabayashi *et al.*, 1983). Three indole compounds, indole-3-acetonitrile, 4-methoxyindole-3-acetonitrile and 4-methoxyindole-3-aldehyde, were isolated as mutagen precursors from fresh Chinese cabbage (Wakabayashi *et al.*, 1985b, 1986). The structures of these six mutagen precursors are shown in Figure 1.

**Fig. 1. Structures of mutagen precursors isolated from soya sauce and Chinese cabbage**



The mutagenicities of these six compounds were tested after treatment with 50 mM sodium nitrite at pH 3.0 for 1 h at 37°C in the dark. The numbers of revertants of TA100 without metabolic activation (S9 mix) induced per mg of mutagen precursor were 3900 for tyramine, 17 400 for (-)-(1*S*, 3*S*)-MTCA, 13 000 for (-)-(1*R*, 3*S*)-MTCA, 17 400 for indole-3-acetonitrile, 31 800 for 4-methoxyindole-3-acetonitrile and 156 900 for 4-methoxyindole-3-aldehyde. The directly-acting mutagenicities of the six precursors with nitrite

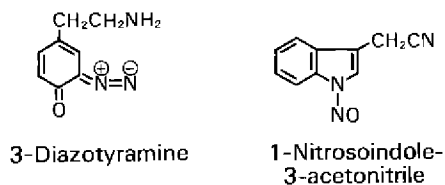
were also observed in TA98, these activities being similar to or less than those in TA100. On addition of S9 mix, the mutagenicities of the MTCA's and indole compounds after nitrite treatment decreased markedly, but that of tyramine changed much less. None of the mutagen precursors themselves was mutagenic to TA100 or TA98 with or without S9 mix.

### Determination of the structures of mutagens produced from mutagen precursors with nitrous acid

The structures of the mutagens produced from tyramine and indole-3-acetonitrile on nitrite treatment have been elucidated.

Tyramine (5 mM) was treated with sodium nitrite (50 mM) at pH 1.0 for 1 h at 37°C, and the nitrosation reaction was terminated by addition of ammonium sulfamate. The reaction mixture was then injected into an ODS column for high-performance liquid chromatography (HPLC). Two reaction products, a mutagen and a nonmutagen, were obtained. From the physical and chemical properties of the mutagen, its structure was deduced to be 4-(2-aminoethyl)-6-diazo-2,4-cyclohexadienone (3-diazotyramine), as shown in Figure 2. This deduction was confirmed by comparison of various spectral data for the mutagen with those of synthetic 3-diazotyramine, which was prepared from 3-aminotyramine with nitrous acid. The nonmutagen was identified as 3-nitrotyramine (Ochiai *et al.*, 1984).

**Fig. 2. Structures of 3-diazotyramine and 1-nitrosoindole-3-acetonitrile**



1-nitrosoindole-3-acetonitrile by various spectral analyses, as shown in Figure 2 (Wakabayashi *et al.*, 1985c).

Indole-3-acetonitrile (3.6 mM) was treated with sodium nitrite (50 mM) at pH 3.0 for 1 h at 37°C; then, ammonium sulfamate was added to decompose excess nitrite, and the reaction mixture was extracted with ethyl acetate. The ethyl acetate extract was subjected to HPLC on a silica column. Only one peak other than indole-3-acetonitrile was detected, and this showed directly-acting mutagenicity; the mutagen was found to be

### Biological properties of 3-diazotyramine and 1-nitrosoindole-3-acetonitrile

**Mutagenicity:** The mutagenicities of 3-diazotyramine and 1-nitrosoindole-3-acetonitrile in TA100 and TA98 (Table 1) were similar to that of *N*-methyl-*N*-nitrosourea, which induced 47 000 revertants/mg in TA100 without S9 mix.

The mutagenicities of 3-diazotyramine and 1-nitrosoindole-3-acetonitrile were also examined in Chinese hamster lung cells with diphtheria toxin resistance (DT) as a selective marker. 3-Diazotyramine induced 8300 DT mutants/10<sup>6</sup> survivors per mg without S9 mix, while 1-nitrosoindole-3-acetonitrile induced 15 000 DT mutants/10<sup>6</sup> survivors per mg without S9 mix. These specific activities were similar to those of *N*-methyl-*N*-nitrosourea and *N*-nitrosodimethylamine.

**Carcinogenicity:** The carcinogenicity of 3-diazotyramine was tested in male Fischer 344 rats five weeks old at the start of the experiment. The animals were given 0.1% synthetic 3-diazotyramine hydrochloride in deionized water to drink from a light-proof container throughout the experiment. The 3-diazotyramine solution was prepared freshly every three or four days. About one-third of the 3-diazotyramine in this solution in the light-proof container decomposed in four days. The experiment was terminated at week 116. Tumours were found only in the oral cavity of 19 of the 28 treated rats; no tumour of the oral cavity was found in control rats. Histologically, the tumours were squamous-cell carcinomas originating in the epithelium of the floor of the mouth, close to the root of the tongue (Nagao *et al.*, 1986).

**Table 1. Mutagenicities of 3-diazotyramine and 1-nitrosoindole-3-acetonitrile in *S. typhimurium* TA100 and TA98**

Compound	Revertants/mg			
	TA100		TA98	
	-S9 mix	+S9 mix <sup>a</sup>	-S9 mix	+S9 mix <sup>a</sup>
3-Diazotyramine	112 000	98 000	72 000	32 000
1-Nitrosoindole 3-acetonitrile	45 000	3000	30 000	1000

<sup>a</sup>Containing 100  $\mu$ l of 9000  $\times$  g supernatant prepared from polychlorinated biphenyl-treated rat livers, in a total volume of 500  $\mu$ l

**Table 2. Amounts of tyramine in various foods**

Food	Tyramine ( $\mu$ g/g or /ml)
Soya sauce	14.0 - 2250
Soya bean paste	0.21 - 169.5
Cheese	29.8 - 953
Meat extract	95 - 304
Beer	1.06 - 1.30

sor (Wakabayashi *et al.*, 1983, 1985a). Japanese *sake* also contains MTCA (Sato *et al.*, 1975).

**Indoles:** The yields of indole-3-acetonitrile, 4-methoxyindole-3-acetonitrile and 4-methoxyindole-3-aldehyde from 300 g fresh Chinese cabbage were 80, 60 and 720  $\mu$ g, respectively (Wakabayashi *et al.*, 1986). Indole-3-acetonitrile, which is a plant growth hormone, is also reported to be present in cabbage (Henbest *et al.*, 1953) and bean sprouts (Okamoto *et al.*, 1967). 4-Chloro-6-methoxyindole has been isolated from fava beans as a mutagen precursor, at a yield of about 20  $\mu$ g/kg (Yang, D. *et al.*, 1984).

In addition, the very widespread indole compounds tryptophan and tryptamine have also been shown to become mutagenic after nitrite treatment (Ohta *et al.*, 1981). We recently tested the mutagenicities of 31 indole compounds after treatment with nitrite and found that 22 were mutagenic (Ochiai *et al.*, 1986). Of these, 17 are present in the human environment. 1-Methylindole and 2-methylindole, which are constituents of cigarette smoke, showed marked mutagenicity after nitrite treatment.

### Occurrence of mutagen precursors

**Tyramine:** We measured the amounts of tyramine in 22 kinds of soya sauce produced in Japan, the USA, the Republic of Korea and the Philippines. All of the soya sauces tested, except three produced in the USA, contained tyramine at 14-2250  $\mu$ g/ml, as shown in Table 2 (Ochiai *et al.*, 1984; Wakabayashi *et al.*, 1984, 1985a). Most soya sauces produced in Japan contained higher amounts of tyramine than those produced in other countries.

Tyramine has also been reported to be present in various other foods, as shown in Table 2 (Blackwell & Mabbitt, 1965; Yamamoto *et al.*, 1980; Smith, 1981). Cheeses contain high concentrations of tyramine, and this is known to be related to the effect of cheese in inducing hypertension in patients taking monoamine oxidase inhibitors.

**MTCA:** Japanese soya sauces contained as much as 82-678  $\mu$ g/ml MTCA, but soya sauces produced in the other countries contained much smaller amounts of this precursor



### Conclusion

Tyramine,  $\beta$ -carboline compounds and indole compounds are widely distributed in our environment. Accordingly, it is very important to elucidate whether directly-acting mutagens are actually formed in the human stomach from these precursors with nitrite. We found that indole-3-acetonitrile reacted rapidly with 1 mM nitrite, which is a physiologically feasible concentration in human saliva, and produced a mutagenic *N*-1-nitroso-substituted compound. Yang *et al.* (1984) also demonstrated that the nitrosation of indoles was a fast reaction. In addition, we detected DNA modification in the glandular stomach of rats, using a  $^{32}\text{P}$ -postlabelling method, when 1-nitrosoindole-3-acetonitrile was given by gastric intubation. These observations suggest that 1-nitrosoindole-3-acetonitrile could be formed from indole-3-acetonitrile and nitrite and cause DNA damage in the human stomach. The rates of nitrosation of  $\beta$ -carboline compounds are probably faster than, or similar to, that of indole-3-acetonitrile. However, the exact nitrosation kinetics of tyramine have not yet been elucidated. In order to estimate the hazard presented by tyramine,  $\beta$ -carboline compounds and indole compounds to humans, study of the exact kinetics of their nitrosation is required. Furthermore, carcinogenicity tests of  $\beta$ -carboline compounds and indole compounds in the presence of nitrite are necessary.

### Acknowledgements

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# ENDOGENOUS FORMATION OF *N*-NITROSO COMPOUNDS: A CURRENT PERSPECTIVE

S.R. Tannenbaum

*Massachusetts Institute of Technology, Department  
of Applied Biological Sciences, 77 Massachusetts Avenue,  
56-311 Cambridge, MA 02139, USA*

**Exposure of people to nitrosating agents occurs through multiple pathways, ranging from nitrogen dioxide reactions in the lung to acid-catalysed nitrosation in the stomach to nitrosation mediated by macrophages or bacteria. The use of *N*-nitrosoproline (NPRO) as an index of endogenous nitrosation has proved to be especially valuable for some of these pathways but may not be universally indicative. Since the development of the NPRO test by Ohshima and Bartsch in 1981, several forms of the test have been used for different purposes. This paper examines some of the issues related to endogenous nitrosation and NPRO and attempts to view the immediate future.**

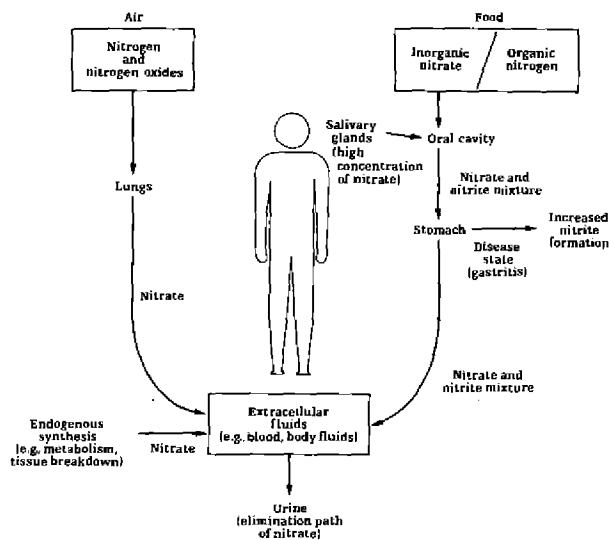
Much has been written on the endogenous synthesis of nitrate, nitrite and *N*-nitroso compounds, and the many papers in this volume dealing with this subject are a tribute to the current vigour of this area of research. This paper is not intended as a review of the field, but as the personal comment of one long-time observer and worker in the field on the past, present and future.

## **Exposure to nitrosating agents**

Figure 1 gives a summary of some of the interlocking pathways of human exposure to nitrosating agents. In considering endogenous formation of *N*-nitroso compounds, this is where we must begin. The formation of nitrite from nitrate in saliva, in the hypochlorhydric stomach and in the infected bladder are well-documented phenomena that have been adequately described in the past. Their connection with bacterially catalysed nitrosation, however, is a subject of great current interest which will receive further comment later in this paper.

The major entry point for nitrate/nitrite into the body is food and water, except in the case of infection and/or inflammation. This latter process is now known to be mediated primarily by macrophages (Stuehr & Marletta, 1985 and this volume) and, most importantly, to involve the formation of nitrite. A limited number of quantitative studies on infected people has indicated that hundreds of millimoles of nitrite could be formed in an individual in a single day (Wagner *et al.*, 1985 and unpublished observations). We have just presented evidence that some of this nitrite can be converted to nitrosamines (Miwa *et al.*, this volume). How much this process might contribute to the endogenous synthesis of *N*-nitroso compounds is unknown. There is also a background level of endogenous formation of nitrate which is not necessarily connected to stimulation of macrophages. This might comprise as much as 200  $\mu$ mol of nitrate/nitrite per day and arise from other

Fig. 1. Nitrate distribution in humans



oxidative processes (Dull & Hotchkiss, 1984b; Saul & Archer, 1984a). The question of whether these processes contribute to *N*-nitrosation is an even deeper mystery. I will come back to these problems when I discuss NPRO.

Another important, but poorly understood, mechanism of exposure is *via* nitrogen oxides in air. This is particularly important to consider for individuals exposed to combustion gases and polluted air. Although estimates of the relative contribution of this source show it to be small relative to total nitrate/nitrite exposure, it may be strongly correlated with nitrosamine exposure, as shown in a recent study (Garland *et al.*, 1986) in which atmospheric nitro-

gen dioxide was positively correlated with urinary *N*-nitrosodimethylamine for a group of individuals over a period of time. The paper of Tsuda and coworkers (this volume) shows how difficult it might be to demonstrate in a population an effect that is readily demonstrable in an individual [*N*-nitrosothiazolidine 4-carboxylic acid (NTCA) and *N*-nitroso(2-methylthiazolidine) 4-carboxylic acid (NMTCA) in smokers and nonsmokers].

What is increasingly apparent is that a nitrate/nitrite balance sheet does not give an accurate picture of the potential for *N*-nitrosation in various tissues and body compartments. Large quantities of nitrite may make only a small contribution to *N*-nitrosation if conditions are unfavourable for a reaction. Conversely, small quantities may play an important role in carcinogenesis in specific tissues.

**Exposure to endogenously formed *N*-nitroso compounds**

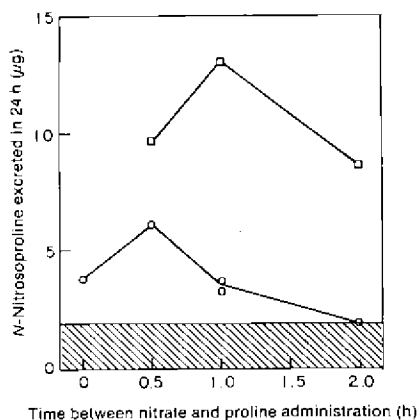
Since Ohshima and Bartsch (1981) developed their NPRO test for endogenous nitrosation, experiments have been carried out in many laboratories measuring urinary excretion of NPRO and other nitrosated amino acids. Most of those laboratories are represented by papers in this volume. Interpretation of all of the available data has not proved to be a simple task. The one immutable conclusion thus far reached is that essentially all humans tested so far have some NPRO in their urine, and that it is undoubtedly of endogenous origin. What is far less clear is the relation between this index and disease. I would like to discuss some aspects of sources of variability in this test and comment upon its adequacy to measure various possible routes and mechanisms of *N*-nitrosation.

There are several ways of conducting the NPRO test in laboratories and epidemiological studies:

### Form 1 (nitrate + proline)

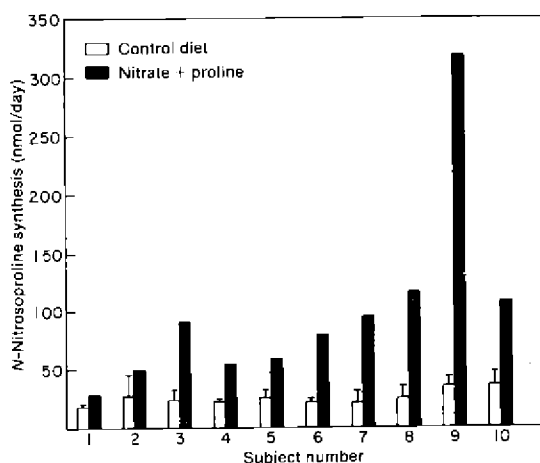
This is the original form of the test, and the one that is subject to the least number of variables. The system is swamped with 'nitrosator' and 'nitrosatee', so that the amount of NPRO is dependent upon other factors. An extremely important variable is the temporal relationship for various reactants and catalysts in the reactive compartment (presumably the stomach). The administered nitrate must be distributed through the body, taken up into saliva, converted to nitrite, and swallowed prior to the reaction. Individual differences in this process of nitrate distribution, conversion to nitrite and stomach emptying will be important determinants of NPRO yield. This is illustrated graphically for two people in Figure 2, where peak values for NPRO formation occur at 0.5 h between doses of nitrate and proline for one individual and at 1 h for the other. These and other factors (e.g., inhibitors and catalysts) will determine the overall amount of excreted NPRO, and an experiment to determine the extent of this variability was conducted. As shown in Figure 3, individuals on a controlled diet excrete a low, fairly uniform, level of NPRO in the absence of nitrate and proline. Administration of nitrate and proline leads to significant but highly variable results, with a greater than ten-fold variation between some individuals. Similar data are shown in another paper in this volume (Leaf *et al.*).

**Fig. 2.** Effect of time between doses of nitrate and proline on formation of NPRO



The conditions of the experiment are otherwise as described by Wagner *et al.* (1985).

**Fig. 3.** Excretion of NPRO by individuals on a controlled diet and after receiving nitrate and proline



The conditions of the experiment are as described by Wagner *et al.* (1985).

### Form 2 (proline)

This form of the test is currently being used in epidemiological studies (Lu *et al.*, 1986). It is designed to test the capacity of an individual for nitrosation of an amine, in this case, proline. It is obviously subject to all of the variables of Form 1 of the test, but, as far as I know, this variation of the test has not been studied under controlled laboratory conditions. It does reveal interesting differences between populations, which presumably reflect gastric nitrosation potential (see several papers, this volume). The limitations of proline as a test amine are discussed later in this paper.

*Form 3 (no nitrate or proline)*

This form of the test has been employed to determine the distribution of NPRO synthesis in populations on uncontrolled diets. The amount of NPRO excreted is a function of endogenous synthesis and dietary content. In a fairly large study of this type conducted at the Roche laboratories in Nutley, NJ, USA (Garland *et al.*, 1986), we have analysed urinary NPRO, NTCA and nitrate in 24 individuals for 20 daily collections. Some important conclusions of this study (in collaboration with W. Willet of Harvard University) are as follows:

- (1) Within-person variation is greater than between-person variation for NPRO and NTCA.
- (2) Between-person variation is greater than within-person variation for nitrate.
- (3) NPRO level is not correlated with nitrate level, and NTCA level is correlated only marginally with nitrate level.
- (4) Diet is a significant contributor to urinary NPRO.
- (5) There is a more than ten-fold variation in urinary NPRO levels in this population.

The fact that all three forms of this test have great variability should not be interpreted as diminishing the value of the information. Rather, we should ask whether the existence of these large differences doesn't tell us something about the variability of nitrosation potential in any population and whether this might be a clue to differences in disease potential.

**Limits of nitrosamino acids as indices of endogenous nitrosation**

I have already discussed various mechanisms of endogenous nitrosation. How good are NPRO and NTCA as indices for these mechanisms, and how well do they reflect the extent of nitrosation of other *N*-nitroso compounds? Studies on the incorporation of <sup>15</sup>N-nitrate into NPRO have demonstrated that there are at least two pools of NPRO in the body (Wagner *et al.*, 1985). One of these pools incorporates <sup>15</sup>N and is modulated by ascorbic acid, while the other does not. The first of these pools probably represents gastric synthesis, while the second represents mammalian cell-mediated synthesis and/or nitrogen oxide-mediated synthesis. In the absence of disease, endogenous nongastric synthesis is fairly uniform (Fig. 3), so the differences between individuals may be ascribed to gastric synthesis. Thioproline nitrosates about 1000 times faster than proline and probably *via* a different mechanism (Tahira *et al.*, 1984), but the concentration of thioproline in various body compartments is unknown, and dependent upon its own precursors. Therefore both NPRO and NTCA together may be useful for assessing only some types of endogenous nitrosation. A question remains as to whether these indices are of value for indicating differences in the population for cell-mediated synthesis, including both macrophages (Miwa *et al.*, this volume) and bacteria (Calmels *et al.*, this volume; Leach *et al.*, this volume; O'Donnell *et al.*, this volume).

**The gastric cancer hypothesis**

Several papers in this volume have raised the issue of whether high levels of nitrite in the hypochlorhydric stomach necessarily lead to elevated synthesis of *N*-nitroso compounds. The paradox of nitrite stability at high pH and nitrite reactivity at low pH has long been the *bête noire* of the gastric cancer hypothesis (Correa, this volume). The low values found for urinary NPRO in high-risk gastric cancer areas (Lu *et al.*, this volume) and in high-risk patients (Hall *et al.*, this volume) argue against nitrosation as a causative factor, in spite of the correlation of high gastric nitrite with increased risk at a number of tumour sites (Caygill *et al.*, this volume).

The critical question, therefore, is the adequacy of NPRO as a nitrosation index for bacterially-mediated nitrosation. As a charged molecule, it may not be capable of entering cells and therefore might not represent the nitrosation potential for more neutral or more lipophilic molecules. Thus, the measurement of NPRO might not be a test of the gastric cancer hypothesis; it might instead be a test of the adequacy of the methodology to test the hypothesis.

#### **The case of the disappearing nitrosamines**

It is only five years since the original publication of the NPRO test. In that time we have learned infinitely more about endogenous nitrosation in people than in the previous 15 years of indirect experimentation. There is undoubtedly a great deal more we can learn from continuing laboratory and population studies of the various forms of the test. However, it is time to begin to confront the 'case of the disappearing nitrosamines'. These are the ones that are metabolized and damage our DNA. Are measurements of excreted DNA adducts a possible means (Shuker, Bailey & Farmer, this volume; Shuker, Howell & Street, this volume; Wild *et al.*, this volume; Belinsky *et al.*, this volume)? Is another possible direction the monitoring of adducts to haemoglobin (Hecht, this volume)? We have bare beginnings of these approaches in 1986. I can hardly wait for the 1989 IARC meeting.

#### **Acknowledgements**

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# ABSENCE OF URINARY *N*-NITROSODIMETHYLAMINE IN FASTING HUMANS FOLLOWING ALCOHOL CONSUMPTION

J.R. Milligan<sup>1</sup>, P.F. Zucker<sup>1</sup>, P.F. Swann<sup>2</sup> & M.C. Archer<sup>1,3</sup>

<sup>1</sup>*Department of Medical Biophysics, University of Toronto,  
Ontario Cancer Institute, 500 Sherbourne Street, Toronto,  
Ontario M4X 1K9, Canada; and* <sup>2</sup>*Courtauld Institute of Biochemistry,  
Middlesex Hospital Medical School, London W1P 7PN, UK*

**Fasted volunteers consumed 350 mg nitrate in beet juice; 1 h later, they consumed a volume of 10% alcohol in carbonated water sufficient to raise blood alcohol concentrations to at least 80 mg/100 ml. This alcohol concentration was then maintained over a 6-h period. During this period, and during the subsequent 12 h, no *N*-nitrosodimethylamine (NDMA) or any other volatile nitrosamine was excreted in the urine of the volunteers (detection limit, 0.01  $\mu$ mol). Therefore, less than 0.5  $\mu$ mol NDMA is likely to be present in the stomach of a fasting human at any time.**

To test the hypothesis that NDMA, and possibly other carcinogenic nitrosamines, is synthesized from endogenous substrates in the gut, urinary excretion of nitrosamines has been measured in people administered ethanol to inhibit metabolism (Swann *et al.*, 1984). Recently, Spiegelhalter and Preussmann (1985) used ethanol to inhibit metabolism of NDMA formed in subjects consuming amidopyrine and nitrate. The results of their control experiments, however, in which two subjects consumed alcohol and nitrate but no amidopyrine, were equivocal. In one case, there was no detectable NDMA in the urine, while in the other 0.1  $\mu$ g/l was found (detection limit, 0.05  $\mu$ g/l).

## Experimental design

Five healthy male volunteers (22-42 years old) were used, who consumed typical North American diets; one volunteer smoked cigarettes. For 12 h prior to and during the experiment, no food or beverages, except as specified, were consumed by these individuals. To start the experiment, they consumed 235 ml of beet juice containing 325 mg nitrate; 1 h later, after providing a saliva sample, they consumed 10% v/v ethanol in carbonated water to raise their blood alcohol concentrations to 80-100 mg/100 ml. Approximately every half-hour for 6 h, blood alcohol concentrations were measured, and additional volumes of alcohol were consumed so that, for four volunteers, blood alcohol levels did not fall below 80 mg/100 ml; one individual was able to maintain a blood alcohol level of only about 50 mg/100 ml. Urine was collected in bottles protected from light that contained 1 g sodium azide to remove any nitrite that may have been present or formed during storage, and 100  $\mu$ l morpholine to detect artefactual nitrosamine formation during analysis. Urine was also collected over the next 12 h.

<sup>3</sup> To whom correspondence should be addressed at: The Beatson Institute for Cancer Research, Wolfson Laboratory for Molecular Pathology, Garscube Estate, Switchback Road, Bearsden, Glasgow G61 1BD, Scotland, UK (up to 30 June 1987).

The nitrate concentration of beet juice, and the nitrite concentration of saliva samples were determined as described previously (Saul & Archer, 1983; Ladd *et al.*, 1984b). After urine samples had been made up to 3 litres with water, KCl was added to a concentration of 1 M. The samples were extracted with methylene chloride ( $5 \times 100$  ml), the extracts were combined, dried over magnesium sulfate, concentrated, and analysed by gas chromatography using a 10% Carbowax 20 M column with a Thermal Energy Analyzer as detector. *N*-Nitrosodiethylamine (10  $\mu$ g) was used as an internal standard. Recoveries of NDMA, *N*-nitrosodiethylamine and *N*-nitrosomorpholine were 67, 71 and 78%, respectively.

### Analytical measurements

Salivary nitrite concentrations 1 h after beet juice consumption were in the range 0.56-2.24 mM, comparable to our previous findings using the same protocol (Ladd *et al.*, 1984). Analysis of the urine samples excreted during the 6-h period of alcohol consumption, or during the subsequent 12 h, showed no evidence of NDMA or any other volatile nitrosamine (detection limit, 0.01  $\mu$ mol; there was no evidence of artefactual nitrosamine formation during the analytical procedures).

### Estimation of maximum extent of endogenous nitrosation

Rats under conditions of alcohol intoxication excreted in urine about 2% of a dose of 30  $\mu$ g/kg NDMA administered by gavage. This value agrees very well with the estimated excretion of up to 0.6  $\mu$ mol (30  $\mu$ g) NDMA in beverages containing small amounts of alcohol (Speigelhalder *et al.*, 1982). Assuming, therefore, that 2% of NDMA formed is excreted in urine, our current experiments suggest that less than 0.5  $\mu$ mol of NDMA formed by nitrosation of endogenous substrates is likely to be present in the human stomach at any time. Experiments with rats, however, have shown that the urinary bladder is permeable to NDMA (Swann, unpublished observations), so that, if humans are like rats in this respect, and if the equilibration of NDMA in urine with total body water is rapid, then the amount of NDMA formed in the stomach would actually be much higher and still have remained undetected in our experiments.

Our finding of no NDMA in the urine of humans administered alcohol does not preclude endogenous formation of *N*-nitroso compounds that are (a) not sufficiently stable to be excreted in urine, (b) metabolized at a high rate even in the presence of high blood alcohol concentrations, (c) formed from endogenous precursors the biosynthesis of which is inhibited by alcohol or (d) formed from exogenous precursors or (e) formed more rapidly than NDMA.

### Acknowledgements

This work was supported by the Ontario Cancer Treatment and Research Foundation and the National Cancer Institute of Canada.



# RELATIONSHIP BETWEEN ASCORBIC ACID DOSE AND *N*-NITROSOPROLINE EXCRETION IN HUMANS ON CONTROLLED DIETS

C.D. Leaf, A.J. Vecchio, D.A. Roe & J.H. Hotchkiss<sup>1</sup>

*Institute of Food Science and Division of Nutritional Science,  
Department of Food Science, Cornell University,  
Ithaca, NY 14853, USA*

A logarithmic dose-response relationship between ascorbic acid dose and *N*-nitrosoproline (NPRO) excretion in humans on a controlled diet was established. Seven healthy males were placed on a low-nitrate, low-ascorbic acid diet for 12 consecutive days and given nitrate on days 3-12 and L-proline on days 4-12, after the nitrate dose. Ascorbic acid was given in increasing amounts with the proline on days 5-10. Urine was analysed quantitatively for nitrate, NPRO and ascorbic acid. Ascorbic acid doses as low as 0.05 mmol reduced NPRO excretion by an average of 6 nmol/day; however, as much as 5.68 mmol ascorbic acid did not return NPRO excretion to levels observed before nitrate and proline were administered. Complete inhibition of endogenous NPRO formation from exogenous precursors requires more than the 2:1 molar ratio of ascorbic acid to nitrite that has been demonstrated *in vitro*. These data may be useful in interpreting epidemiological studies of nitrate exposure and in making dietary recommendations.

Endogenous formation of *N*-nitroso compounds may be important in the etiology of certain human cancers such as gastric and oesophageal cancers (Mirvish, 1983; Lu *et al.*, 1986). Several factors influence the endogenous formation of *N*-nitroso compounds both qualitatively and quantitatively, including the type and quantity of amine/amide precursors in the diet (Mirvish, 1975), levels of dietary nitrate and atmospheric NO exposure (Mirvish *et al.*, 1981; Wagner *et al.*, 1983a), salivary nitrate reductase activity (Spiegelhalter *et al.*, 1976), gastric pH (Kim *et al.*, 1982), microbial colonization of the stomach, and the presence of nitrosation catalysts and inhibitors in the diet (Archer, 1984; Wagner *et al.*, 1985). Ascorbic acid is an influencing factor of particular interest because it can inhibit nitrosation both *in vitro* (Archer *et al.*, 1975) and *in vivo* (Mirvish *et al.*, 1973a) and may be a protective factor for gastric cancer (Mirvish, 1983).

## Study design and execution

The subjects were seven nonsmoking males, aged 22-34, judged to be in good health by a physical examination including routine blood and urine screening, and were not taking any medications or vitamin supplements. During the 12 consecutive days of the study, subjects consumed a low-nitrate, low-ascorbic acid standardized diet prepared in a human metabolic unit (Table 1). They drank only distilled water *ad libitum* and no alcoholic beverage. The

<sup>1</sup> To whom correspondence should be addressed

diet was analysed for nitrate and NPRO and found to contain 0.46 mmol/day and 11.7 nmol/day, respectively. A daily diary was maintained by each subject to record any possible deviation from the protocol and any variation in health, of which there was none.

**Table 1. Controlled diet**

Meal	Item	Amount
Breakfast	Shredded-wheat cereal	41 g
	Whole milk	240 g
	Wheat bread	59 g
	Margarine	5 g
	Jelly	19 g
	Coffee or tea	1-2 cups
	Lunch	*Peanut butter/jelly/wheat bread
*Shortbread cookies		48 g
*Ginger ale		368 g
Dinner	Chicken breast (boneless, skinless)	145 g
	*Rice	178 g
	Roll	32 g
	Margarine	5 g
	Whole milk	240 g
	Vanilla ice cream	86 g
Evening snack	*Bagel	100 g
	*Cream cheese	29 g
	*Clear soda	368 g

Basic diet contained 2700 calories/day; subjects were allowed to choose extra portions of items marked \* on days 1 and 2, and then continued with the same amount for the remainder of the study.

were collected twice daily and stored at 5°C until the 24 h period was completed. For each 24-h collection, total volume, specific gravity and creatinine content were measured to ensure subject compliance. One-litre aliquots were retained for analysis. NPRO and 2,6-dimethylmorpholine were added to some samples as controls. Phenylmercuric acetate (10 mg) was added to prevent microbiological activity, and the pH adjusted to 5 for storage at -5°C.

### Analytical methodology

NPRO was extracted from urine by a modification of the method of Wagner *et al.* (1985). The samples were derivatized to the methyl ester with boron fluoride-methanol in a manner similar to that described by Ladd *et al.* (1984b). <sup>15</sup>N incorporation was determined by gas chromatography-mass spectrometry, using a modification of the method of Wagner *et al.* (1985). NPRO in the diet was determined by the method of Sen *et al.* (1983). Storage controls showed no loss of NPRO and no gain of *N*-nitroso-2,6-dimethylmorpholine during the entire storage period.

On days 3-12, the diet was supplemented with a bolus dose of sodium nitrate (5.24 mmol). On one day, six individuals received <sup>15</sup>N-nitrate. The nitrate was administered in mid-afternoon, at least 2 h after the subjects had last eaten, in 10 ml distilled water and followed by 100 ml distilled water. On days 4-12, a bolus dose of L-proline (4.35 mmol in 25 ml distilled water) was administered 30 min after the nitrate dose and followed by 100 ml distilled water. Ascorbic acid (donated by Hoffmann-La Roche Inc., Nutley, NJ, USA) was given in increasing amounts (0.01, 0.05, 0.26, 0.52, 2.62, 5.68 mmol) in 10 ml distilled water with the proline on days 5-10.

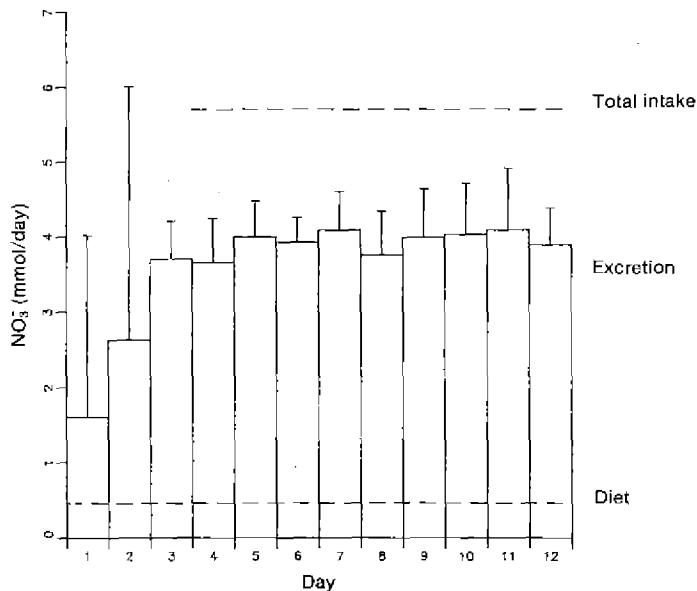
All urine was collected 24 h after dosing. Subjects urinated directly into 1-litre opaque Nalgene bottles containing 5 ml ammonium sulfamate solution (0.2 mg/ml 3.6 N sulfuric acid). Bottles

Nitrate in urine was analysed by conversion to nitrobenzene with subsequent detection by gas chromatography-thermal energy analysis, as described by Dull and Hotchkiss (1984c). Nitrotoluene was added as an internal standard. Nitrate in the diet was determined by the method of Ross and Hotchkiss (1985). Total ascorbate (ascorbate plus dehydroascorbate) in urine was determined as described by Okamura (1981). Urinary ascorbate was not detectable on days 2 and 11 when no ascorbic acid was given.

### Nitrate excretion

The average daily nitrate intake from diet and dose on days 3-12 was 5.7 mmol, 8.1% of which was from the diet. During this period, excretion of both dietary and endogenously formed nitrate averaged  $3.9 \pm 0.16$  mmol/day or 68.7% of the daily nitrate intake (Fig. 1). On days 1 and 2, the variation between subjects with regard to nitrate excretion was large (150% and 129%, respectively), due, perhaps, to a large variation in dietary habits before the subjects began the controlled diet. Even with the added nitrate, two subjects decreased their nitrate excretion after two days on the study. Other nitrate balance studies have shown that an average of 60-70% of a large oral dose is excreted by humans within 48 h (Wagner *et al.*, 1983a; Bartholomew & Hill, 1984). In agreement with earlier findings (Wagner *et al.*, 1983a), we observed that the ascorbic acid dose had no effect on nitrate excretion.

Fig. 1. Average intake and excretion of nitrate (plus standard deviation) during the controlled diet period



### NPRO excretion

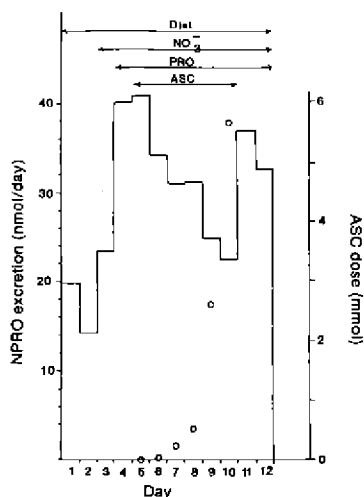
Subjects excreted more NPRO than could be accounted for by intake of preformed NPRO in the diet: average NPRO excretion was never less than  $14 \pm 4$  nmol/day, even when subjects were on the controlled diet with no supplementary nitrate or proline (Fig. 2). Basal NPRO levels, unaffected by large doses of ascorbic acid, of  $26 \pm 10$  nmol/day have been reported (Wagner *et al.*, 1985). When subjects were given both nitrate and proline, average NPRO excretion increased from 14 to 40 nmol/day. Incorporation of  $^{15}\text{N}$  into NPRO demonstrated that the increase was a result

of the nitrate dose and confirms work of Wagner *et al.* (1985). NPRO levels decreased as ascorbic acid doses increased, yielding a linear log dose *versus* NPRO excretion relationship (Fig. 3). Regression analysis produced the equation  $\text{NPRO (nmol/day)} = 23.9 - 5.96 \log [\text{ascorbic acid (mmol)/nitrate (mmol)}]$  with  $r^2 = 0.96$ . The equation predicts that complete inhibition of NPRO formation would require extremely large quantities of ascorbic acid. In this study, as little as 0.05 mmol ascorbic acid decreased NPRO formation by an average of 6 nmol/day. However, as much as 5.68 mmol did not return NPRO excretion to the levels

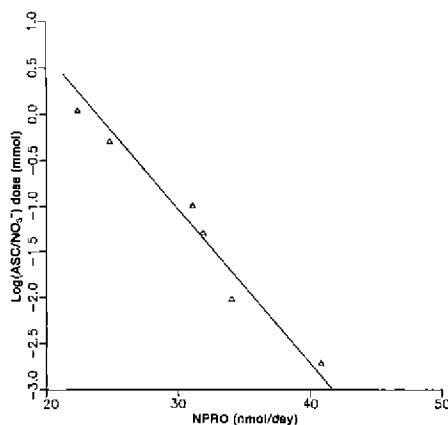
observed before nitrate and proline were administered; this quantity is more than ten times the amount of ascorbic acid necessary to completely inhibit NPRO formation *in vitro*, assuming a 5% reduction of nitrate to nitrite (Mirvish *et al.*, 1972; Fan & Tannenbaum, 1973).

At least three explanations are possible: (1) nitrosation may be occurring at sites where ascorbic acid is not available in sufficient amounts; (2) ascorbic acid may be rapidly lost or otherwise degraded; for example, ascorbic acid is much less effective in the presence of oxygen (Archer *et al.*, 1975); and (3) catalysts, including salivary and gastric thiocyanate may be overcoming the inhibitory effects of ascorbic acid. An *in-vitro* model, based on a method described elsewhere (Mirvish *et al.*, 1973b), demonstrated that at equimolar concentrations of thiocyanate and ascorbic acid, thiocyanate catalysis overcomes ascorbic acid inhibition of NPRO formation below pH 3 (Fig. 4).

**Fig. 2.** NPRO excretion, following administration of 5.24 mmol sodium nitrate, 4.35 mmol L-proline, and 0.01, 0.05, 0.26, 0.52, 2.62 and 5.68 mmol ascorbic acid (ASC) (o), as indicated



**Fig. 3.** Plot of log [ascorbic acid (ASC)/nitrate] dose (mmol) versus NPRO excretion

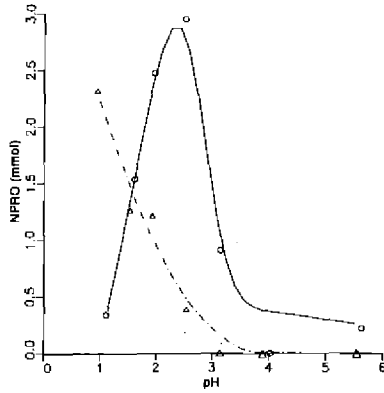


Individuals on a controlled diet vary widely in their ability to form NPRO endogenously from the same amounts of precursors (Fig. 5). In particular, one subject excreted seven times more NPRO than another. Individual differences in oral microflora, gastric pH, and salivary thiocyanate levels are only a few of the many factors influencing endogenous NPRO formation and, possibly, influencing individual cancer risk.

## Conclusion

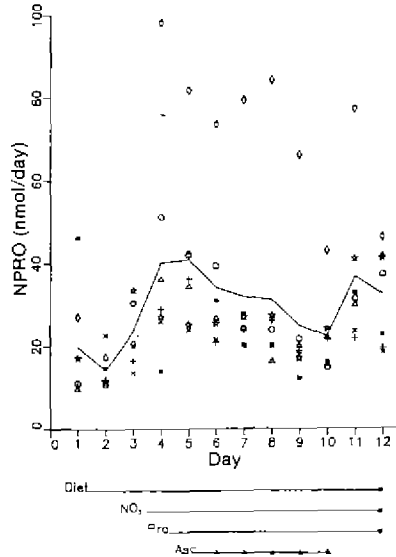
A logarithmic dose-response relationship between ascorbic acid dose and NPRO excretion in humans on defined controlled diets was established. The amount of ascorbic acid required to return NPRO excretion to baseline levels was over ten times the amount predicted by *in-vitro* experiments. This is presumably due to the complexity of the *in-vivo* situation, as suggested by Kim *et al.* (1982). Many factors need to be considered when estimating human risk from exposure to *N*-nitroso compounds and their precursors.

**Fig. 4. In-vitro model of NPRO formation**



Solid line, NPRO formed from 12.5 mmol sodium nitrate and 155 mmol L-proline; broken line, NPRO formed from 12.5 mmol sodium nitrate, 155 mmol L-proline, 25 mmol ascorbic acid and 25 mmol potassium thiocyanate. No detectable NPRO was formed in the presence of 12.5 mmol sodium nitrate, 155 mmol L-proline and 25 mmol ascorbic acid.

**Fig. 5. NPRO excretion during intake of a controlled diet**



Symbols represent individuals, line represents average; Pro, proline; Asc, ascorbic acid

**Acknowledgements**

This material is based upon work supported under a National Science Foundation Graduate Fellowship. The investigation was supported, in part, by PHS Grant 1RO1 CA40833-01 awarded by the National Cancer Institute, DHHS. We would like to thank Heidi Kalkwarf for assistance in developing the diet and managing the human metabolic unit, and John Thurtell for assistance with graphics.

# FORMATION OF *N*-NITROSOIMINODIALKANOIC ACIDS AND THEIR UNSUITABILITY AS BIOLOGICAL MONITORS FOR ENDOGENOUS NITROSATION OF DIPEPTIDES

A.R. Tricker & R. Preussmann

*Institute of Toxicology and Chemotherapy,  
German Cancer Research Centre, Im Neuenheimer Feld 280,  
D - 6900 Heidelberg, Federal Republic of Germany*

**Nitrosation of dipeptides that do not contain imino amino acids leads to rearrangement and formation of *N*-nitrosoiminodialkanoic acids. The optimal pH is 2.0 (0.8-3.2% yield). Under normal gastric conditions, a maximum yield of 0.1  $\mu$ mol total *N*-nitrosoiminodialkanoic acid would be obtained for a typical dietary intake of 0.1 mol dipeptide. This corresponds to a total concentration of about 20  $\mu$ g *N*-nitrosoiminodialkanoic acid/l gastric juice over a 24-h period. *N*-Nitrosoiminodialkanoic acids are excreted quantitatively in urine when fed by gavage to rats; however, they were not detected in normal human urine. It was concluded that determination of these compounds in human urine is not a suitable method for monitoring endogenous nitrosation of dipeptides.**

Human exposure to endogenous formation of *N*-nitroso compounds has been monitored by measuring *N*-nitrosoproline (NPRO) (Ohshima & Bartsch, 1981), *N*-nitrosothiazolidine-4-carboxylic acid and *N*-nitroso-2-methylthiazolidine-4-carboxylic acid (Tsuda *et al.*, 1984) in urine. In an attempt to find a new biological monitor for endogenous nitrosation, we studied the formation *in vitro* of *N*-nitrosoiminodialkanoic acids from dipeptides (Pollock, 1985), which do not contain imino amino acids, in buffer systems and in gastric juice.

## **Nitrosation of dipeptides and analysis**

Nitrosation of 10 mM/l dipeptide was carried out in sealed vials at 25°C in either 0.5 mL 0.1 M aqueous citrate/hydrochloric acid buffer or fasting gastric juice over a pH range 1.0 to 5.5 using 2 mM sodium nitrite with or without 1.5 mM potassium thiocyanate as a nitrosation catalyst. After 16 h, the reaction was terminated by adjusting to pH 4.0 (50 mg potassium hydrogen phthalate) and nitrite destroyed with hydrazine sulfate (50 mg). The reaction mixture was acidified to pH 1.5 (1 M hydrochloric acid), *N*-nitrosoazetidine-carboxylic acid (NAzCA) (200 ng) was added as an internal standard, and the mixture was extracted with diethyl ether, the extracts combined and dried over anhydrous sodium sulfate, concentrated, derivatized with excess diazomethane and analysed by gas chromatography-thermal energy analysis (GC-TEA). Analysis used 10% OV 17 on Supelcoport (80-100 mesh); helium as carrier gas (30 ml/min); injection-port temperature, 180°C; oven maintained at 90°C (1 min) and then programmed to 210°C at 6°C/min. The retention times (in min) of the methylated products derived from dipeptides and standard compounds were

as follows: L-AlaGly and Gly-L-Ala, each 13.2; L-Ala-L-Val and L-Val-L-Ala, each 15.6; Gly-L-Ileu and L-IleuGly, each 16.5; Gly-L-Leu and L-LeuGly, each 16.1; NAzCA, 10.5; and *N*-nitrosoiminodiacetic acid (NIDA), 13.0. The level of detection was determined as 0.5 ng for all tested standard compounds.

#### Excretion of *N*-nitrosoiminodiacetic acid in rats

Excretion studies were carried out using male Sprague-Dawley rats kept under conventional conditions; following fasting for 24 h, 200  $\mu$ g NIDA in 0.5 ml water were administered by gavage. Animals were given food and water *ad libitum* and kept in metabolic cages for 24-h urine collection. The metabolic cages were washed down with distilled water to make the final volume of urine up to 50 ml. A urine aliquot (15 ml) was made up to 50 ml with distilled water, NAzCA (60  $\mu$ g) was added as an internal standard, and the mixture was extracted with ethyl formate ( $4 \times 25$  ml) in the presence of sodium chloride (8 g) and 20% ammonium sulfamate in 3.6 M sulfuric acid (1 mL). Combined extracts were dried over anhydrous sodium sulfate, concentrated and derivatized with excess diazomethane for analysis by GC-TEA.

#### Nitrosation potential of dipeptides

The pH-dependencies of the formation of nitroso derivatives by the eight tested dipeptides in aqueous citrate/hydrochloric acid buffer are shown in Table 1. An optimal pH of 2.0 was observed for all test compounds. This is lower than the optimum of 2.25 reported for the nitrosation of L-proline and other amines (Mirvish, 1972). Individual pairs of dipeptides containing two different amino acids in either the AB or BA configuration gave identical products on nitrosation at less than 5% yield. In addition, dipeptides containing glycine in the *N*-terminal position gave significantly lower yields than the corresponding C-terminal isomers. Glycylglycine failed to give an *N*-nitroso derivative under the conditions used. Dipeptides containing *N*-terminal leucine, isoleucine and valine also produced a second *N*-nitroso derivative, albeit in 0.01-0.02% yield.

Thiocyanate did not catalyse the nitrosation of dipeptides to *N*-nitrosoiminodialkanoic acids, and the yield of the rearranged *N*-nitroso derivative at typical gastric nitrite conditions of  $10^{-5}$ M was negligible ( $10^{-8}$ M *N*-nitrosoiminodialkanoic acid/0.01 M dipeptide) (Table 2). An identical optimal pH for nitrosation was found during nitrosation in gastric juice; however, the yield of *N*-nitroso derivative was reduced by about 65%.

Following consumption of nitrite-containing foods, elevated concentrations of about  $3 \times 10^{-4}$ M nitrite have been measured in gastric juice (Walters *et al.*, 1979). While these may result in a significantly higher nitrite concentration than used in our predictions, nitrite loss by adsorption from the stomach and reactions with ingested nonamino substrates (nitrosation inhibitors) probably cancel out this transitory high level of nitrite over a 24-h period. From the data in Table 2 (nitrite dependency of nitrosation), it can be predicted that, for a typical dietary intake of 0.1 M dipeptide (Challis *et al.*, 1982), about 20  $\mu$ g *N*-nitrosoiminodialkanoic acid/L gastric juice would be formed over a 24-h period.

#### Excretion of *N*-nitrosoiminodialkanoic acids

Analysis of rat urine for NIDA showed a urinary excretion of 96% (SD = 7.4, n = 3), inferring that NIDA is excreted quantitatively in urine and is not metabolized. Using this method, small losses (typically 5%) are normally encountered due to adsorption of the test compound onto solid material such as faeces and the walls of metabolic cages.

**Table 1. pH-dependency of the formation of *N*-nitrosoiminodialkanoic acids from dipeptides<sup>a</sup>**

pH	Percentage yield of <i>N</i> -nitrosoiminodialkanoic acid/dipeptide							
	GlyAla	AlaGly	GlyLeu	LeuGly	GlyIleu	IleuGly	AlaVal	ValAla
1.0	0.17	1.10	0.08	0.77	0.13	0.84	0.54	0.45
1.5	0.26	1.12	0.17	1.41	0.24	1.03	0.68	0.52
2.0	1.43	3.23	0.80	3.23	0.93	2.85	2.23	2.37
2.5	0.15	1.94	0.18	0.98	0.13	1.59	0.71	0.77
3.0	0.13	0.78	0.13	0.63	0.10	0.69	0.37	0.26
3.5	0.09	0.33	0.10	0.45	0.06	0.37	0.30	0.20
4.0	0.09	0.29	0.08	0.29	0.05	0.22	0.19	0.19
4.5	0.04	0.18	0.05	0.18	0.05	0.16	0.13	0.09
5.0	0.05	0.07	0.04	0.15	0.03	0.11	0.08	0.08
5.5	0.003	0.02	0.009	0.05	0.007	0.05	0.03	0.002

<sup>a</sup>Nitrosation performed in 0.1 M aqueous citrate/hydrochloric acid buffer containing 0.01 M/l dipeptide and 0.5 mol/l nitrite; reaction time, 16 h at 25°C

**Table 2. Nitrite dependency of nitrosation of L-alanylglycine and the catalytic effect of thiocyanate<sup>a</sup>**

Concentration of sodium nitrite (M)	Percentage yield of <i>N</i> -nitroso(carboxymethyl)alanine from L-alanylglycine	
	Without catalysis	Thiocyanate catalysis <sup>b</sup>
1.0	3.0	3.0
10 <sup>-1</sup>	0.16	0.16
10 <sup>-2</sup>	0.02	0.04
10 <sup>-3</sup>	0.003	0.005
10 <sup>-4</sup>	0.0001	0.0002
10 <sup>-5</sup>	0.0001	0.0002

<sup>a</sup>Nitrosation of 0.01 M L-alanylglycine performed in pH 2.0 0.1 M aqueous citrate/hydrochloric acid buffer at 25°C for 16 h using variable nitrite concentrations

<sup>b</sup>Nitrosation in presence of 1.5 mM potassium thiocyanate

<sup>c</sup>Typical gastric nitrite concentration



In blind analyses of human urine (100 ml), using the method for the analysis of rat urine, no *N*-nitroso compound corresponding to known *N*-nitrosoiminodialkanoic acids (based on GC-TEA retention times) was identified. This result is not totally unexpected in view of the predicted total concentration of 20  $\mu\text{g}$  *N*-nitrosoiminodialkanoic acids/l gastric juice formed over 24 h, the level of detection for *N*-nitrosated derivatives and the number of possible different *N*-nitrosoiminodialkanoic acids that could be formed. Excluding the imino amino acids, guanidine, histidine, tryptophan and tyrosine, 136 different *N*-nitrosoiminodialkanoic acids can be formed from dipeptides of naturally occurring amino acids. Peptides containing residues of guanidine, histidine, tryptophan and tyrosine fail to give methylated products sufficiently volatile for analysis.

It can be concluded that the monitoring of *N*-nitrosoiminodialkanoic acids in human urine is not a suitable method for biological monitoring of endogenous nitrosation of peptides. Furthermore, substitution of dipeptides that do not contain imino amino acids for proline, as used in the NPRO test (Ohshima & Bartsch, 1981), is not a viable alternative for monitoring endogenous nitrosation.

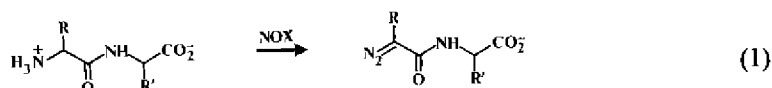
## FORMATION OF DIAZOPEPTIDES BY NITROGEN OXIDES

B.C. Challis<sup>1</sup>, M.H.R. Fernandes, B.R. Glover & F. Latif

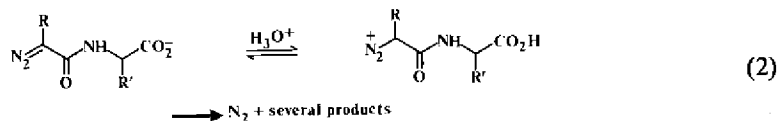
Chemistry Department Imperial College,  
London SW7 2AZ, United Kingdom

A versatile synthesis of diazopeptides is reported and several new compounds described. It is shown that diazopeptides form readily from gaseous nitrogen dioxide in both neutral buffers and human blood. In these media, diazopeptides are sufficiently stable (half-time 0.5-30 h) to qualify as potential circulating carcinogens.

Proteins and peptides are abundant natural substrates which readily undergo nitrosation at the terminal *N*-atom to give a diazo derivative (Equation 1). Diazopeptides were



first isolated by Curtius in 1904, but they remain elusive compounds. Only those derived from glycylopeptides (i.e., *N*-diazoacetyl compounds) and with a protected (e.g., ester or amide) carboxyl terminus are well known. Under the usual acidic conditions for nitrosation, diazopeptides are labile, readily expelling nitrogen (Equation 2).



If diazopeptides were a causal factor in cancer, they would have to be generated under mild, nonacidic conditions. Such conditions may apply to the respiratory tract following the inhalation of nitrogen oxides, as in tobacco smoking. Nitrogen oxides are known to be potent nitrosating agents at  $\text{pH} > 6$  (Challis & Kyrtopoulos, 1979; Challis & Outram, 1982), most are retained on inhalation (Goldstein *et al.*, 1977; Saul & Archer, 1983), and their interaction with erythrocytes is very well known (Kiese, 1974; Doyle & Hoekstra, 1981). Further, most *N*-diazoacetyl peptide derivatives are mutagenic (Banfi *et al.*, 1974; Monti-Bragadin *et al.*, 1974; Pani *et al.*, 1980), and two, *N*-diazoacetyl glycine amide (Brambilla *et al.*, 1972) and *N*-diazoacetyl glycine hydrazide (Brambilla *et al.*, 1970), are pulmonary carcinogens.

<sup>1</sup> To whom correspondence should be addressed

In view of these observations, we investigated the synthesis, analysis and properties of a wider range of diazopeptides. We also examined both their formation from gaseous nitrogen dioxide and their stability in aqueous buffers and in human blood.

### Synthesis of diazopeptides

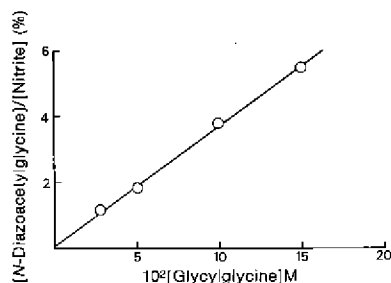
Several new diazopeptides (Table 1) were synthesized by aprotic nitrosation using gaseous nitrogen dioxide. The method is more versatile than procedures (e.g., Looker & Carpenter, 1967) using acidified nitrous acid, because formation of the diazopeptide is faster and decomposition slower. The only constraint is an unreactive, aprotic solvent for the peptide substrate. Unprotected peptides (e.g., glycylalanine, pentaglycine) were therefore solubilized as tetra-alkylammonium salts. The diazopeptides were purified by chromatography on silica and obtained as either oils or solids. Unprotected diazopeptides were obtained by precipitation as calcium salts following chromatography. Yields of purified diazopeptides were about 50% for the ester and amide derivatives and about 20% for the calcium salts.

All of the compounds showed the expected spectroscopic properties (including fast-atom bombardment mass spectrum). For quantification, the most useful property was the strong ultra-violet absorbance (Table 1).

### Formation in aqueous media

Diazopeptides also form readily (half-time, < 1 sec) in aqueous buffers at  $\text{pH} > 6$  on passing gaseous nitrogen dioxide. The yields depended on the peptide concentration and the amount of nitrogen dioxide passed, as shown for glycylglycine in phosphate buffer at  $\text{pH} 6.8$  and  $25^\circ\text{C}$  (Figs 1 and 2). For 0.1 M glycylglycine, about 3.8% of the gaseous nitrogen dioxide reacted with the peptide, the remainder being hydrolysed to a mixture of nitrite and nitrate. The amount of nitrogen dioxide reacting increased with  $\text{pH}$  to reach a maximum of about 11.5% at  $\text{pH} 9.6$ , following the titration curve for the amino group of the peptide. A mechanism consistent with these observations is nitrosation of the unprotonated peptide by the unsymmetrical nitrogen dioxide dimer, followed by rapid deprotonation and dehydration (Scheme 1).

**Fig. 1.** Effect of glycylglycine concentration on the yield of *N*-diazoacetylglycine at  $\text{pH} 6.8$  and  $25^\circ\text{C}$ ; 200 ppm nitrogen dioxide passed for 30 min



**Fig. 2.** Effect of nitrogen dioxide concentration on the yield of *N*-diazoacetylglycine at  $\text{pH} 6.8$  and  $25^\circ\text{C}$ : initial [glycylglycine] = 0.1 M; nitrogen dioxide passed for 30 min

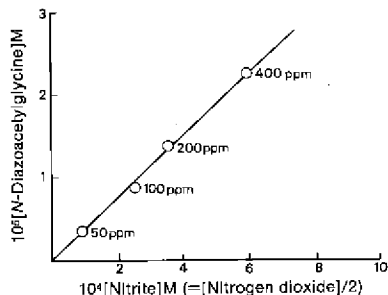
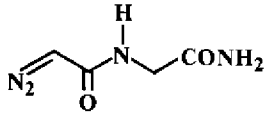
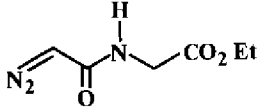
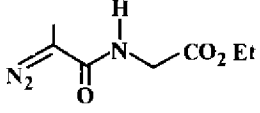
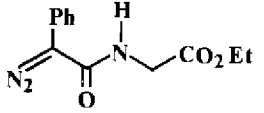
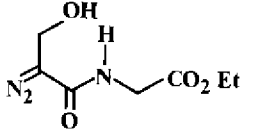
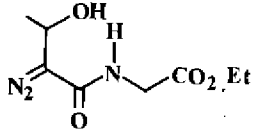
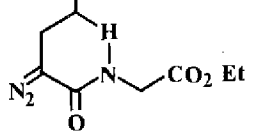
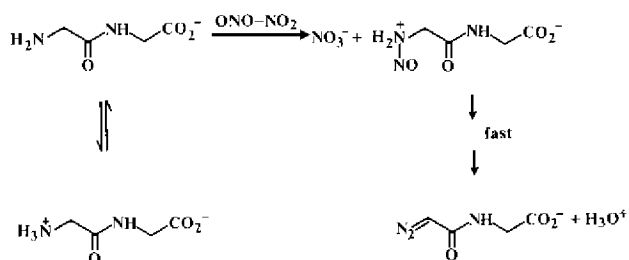


Table 1. Ultra-violet absorption of diazopeptides in water at 25°C

Compound	$\lambda_{\max}$ (nm)	log $\epsilon$
	255	4.432
	250	4.320
	260	3.112
	254	3.372
	255	3.985
	254	4.142
	260	4.170
$(N_2 \text{ digly}^-)_2 \text{ Ca}^{++}$	252	4.345
$(N_2 \text{ trigly}^-)_2 \text{ Ca}^{++}$	250	4.314
$(N_2 \text{ pentagly}^-)_2 \text{ Ca}^{++}$	250	4.257

Scheme 1



Apart from small differences related to the basicity ( $\text{pK}_a$ ) of the terminal amino group, other, structurally different peptides gave similar yields of diazo products to those for glycylglycine. Some of these other diazo peptides, however, decomposed more rapidly (see below). As nitrosation by nitrogen dioxide must involve a dimeric reactant (Challis & Kyrtopoulos, 1979), diazo peptide formation ought to diminish with dilution of nitrogen dioxide. For 0.1 M glycylglycine in aqueous buffer at pH 8.35 and 25°C, this effect was significant only for < 20 ppm nitrogen dioxide (Fig. 3). Even at the 2 ppm level, 2.3% of the gaseous nitrogen dioxide formed *N*-diazoacetyl glycine under these conditions.

#### Stability of diazo peptides in aqueous media

Diazo peptides have a reputation for being unstable. Kinetic studies using ultra-violet spectrophotometry showed that their stability depended primarily on acid catalysts and substituents adjacent to the diazo group.

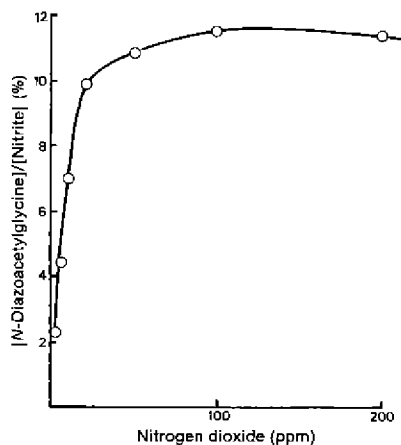
In aqueous buffers ( $\text{HA} \rightleftharpoons \text{A}^- + \text{H}_3\text{O}^+$ ), decomposition followed equation 3

$$\text{Rate} = [\text{Diazo peptide}] \{k_{\text{H}_2\text{O}} + [\text{H}_3\text{O}^+] + k_{\text{HA}} [\text{HA}]\} \quad (3)$$

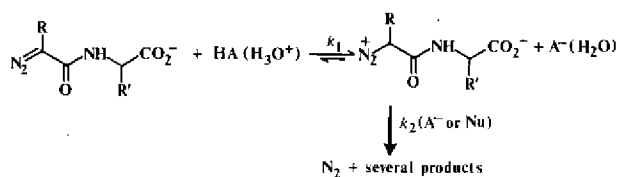
and was therefore general-acid catalysed. For most diazo peptides, equation 3 reflected decomposition *via* an A-SE2 pathway in which  $\text{H}^+$ -transfer (step  $k_1$  of Scheme 2) was rate-limiting. For *N*-diazoacetyl peptides (i.e., from glycyl peptides), however, equation 3 reflected an A-2 pathway in which bimolecular decomposition of the protonated intermediate (step  $k_2$  of Scheme 2) was rate-limiting. Consequently, the latter reactions were also catalysed by nucleophiles such as  $\text{SCN}^-$  and  $\text{Cl}^-$ .

All of the diazo peptides decomposed very rapidly (half-time, < 1 sec) at normal gastric pH, but were relatively stable (half-time = 0.5-30 h) at cellular pH. Pseudo-first-order rate coefficients (rate =  $k$ [diazo peptide]) at pH 7.5 and 25°C (Table 2) show that electron-donating substituents adjacent to the diazo group decrease stability and *vice versa*. Thus, the diazo derivatives of glycyl, seryl and threonyl peptides should be more stable than those of alanyl and phenylalanyl peptides.

**Fig. 3.** Effect of dilution on the % conversion of nitrogen dioxide to *N*-diazooacetylglycine at pH 8.35 and 25°C; initial [glycylglycine] = 0.1 M



### Scheme 2



### Formation and decomposition in human blood

Gaseous nitrogen dioxide interacted rapidly to form mainly nitrite in both serum and plasma, and nitrate in whole blood. No low molecular weight diazopeptides were detected in these solutions; diazopeptides were formed, however, from peptides added to serum and whole blood. The yields were similar to those observed for aqueous buffers, as shown for 0.1 M peptide and 200 ppm nitrite at 25°C in Table 3. Apparently, blood neither inhibits the formation nor accelerates the decomposition of diazopeptides.

The second of these conclusions was confirmed by measuring the decomposition (Rate —  $k$ [diazopeptide]) of authentic diazopeptides in serum and plasma at 25°C (Table 2). Most are more stable in serum and plasma than in aqueous buffer at the same pH and temperature; *N*-diazopropanoyl-L-alanine, for example, is about 40 times more stable. The decomposition rates for serum shown in Table 2 correspond to a half-time of 28 h for *N*-diazooacetylglycine and of 0.5 h for *N*-diazopropanoyl-L-alanine.

**Table 2. Decomposition of diazopeptides at 25°C in phosphate buffer (pH 7.5), serum (pH 7.2-7.6) and plasma (pH 7.2). Initial [diazopeptide], about 10<sup>-4</sup>M**

Diazopeptide	10 <sup>5</sup> k s <sup>-1a</sup>		
	Buffer	Serum	Plasma
	2.12	2.78	
	0.45		
(N <sub>2</sub> trigly <sup>-</sup> ) <sub>2</sub> Ca <sup>++</sup>	0.43		
(N <sub>2</sub> pentagly <sup>-</sup> ) <sub>2</sub> Ca <sup>++</sup>	0.41	2.14	1.95
	1.72	0.68	
	1.47		
	2.58		
	815	23.8	37.9
	8.91	43.3	
	184	5.56	

<sup>a</sup>Rate = k[diazopeptide]

**Table 3. Formation of diazopeptides in phosphate buffer, serum and blood from 0.1 M peptide and 200 ppm nitrogen dioxide at 25°C**

Peptide	Medium	pH	[Diazopeptide]
			$\Sigma$ [nitrite] + [nitrate] <sup>a</sup>
Glycylglycine	Serum	7.1	0.068
Glycylglycine	Buffer	7.4	0.069
Glycylglycine ethyl ester	Serum	6.8	0.060
Glycylglycine ethyl ester	Buffer	7.3	0.058
Glycyl-L-leucine	Serum	7.2	0.074
Glycyl-L-leucine	Blood	7.1	0.068
Glycyl-L-leucine	Buffer	7.6	0.068

<sup>a</sup> $\Sigma$  [nitrite] + [nitrate] = [nitrogen dioxide]

### Conclusions

Although some diazopeptides are respiratory carcinogens (Brambilla *et al.*, 1970, 1972), their endogenous formation has not previously been considered as a causative factor in cancer. Our results suggest that such compounds could form from inhaled nitrogen dioxide in the blood, and probably in saliva. Diazopeptides decompose rapidly at normal gastric pH, but they are sufficiently stable in blood to act as circulating carcinogens.

### Acknowledgements

We thank the Cancer Research Campaign, the Ministry of Agriculture, Fisheries and Food and the Science and Engineering Research Council for support of this work.



**LIPIDIC NITROSATING AGENTS PRODUCED FROM  
ATMOSPHERIC NITROGEN DIOXIDE  
AND A NITROSAMINE PRODUCED *IN VIVO*  
FROM AMYL NITRITE**

S.S. Mirvish, M.D. Ramm, D.M. Babcock, J.P. Sams & M. Panigot

*Eppley Institute for Research in Cancer, University of Nebraska  
Medical Center, Omaha, Nebraska 68105, USA*

In studies on nitrosating agent(s) formed in skin of mice exposed to nitrogen dioxide, we showed that: (i) *N*-nitrosomethylaniline was produced in skin of mice exposed to nitrogen dioxide and then painted with *N*-methylaniline; (ii) a nitrosating precursor in methyl linoleate is associated with peroxidation products; (iii) cholesterol is a major nitrosating precursor in mouse skin, probably because it produces the nitrosating agent, cholesteryl nitrite; (iv) cholesteryl nitrite enhances autoxidation of lipids *in vivo* and on mouse skin and, like sodium nitrite, catalyses the autoxidation of iodide; (v) *N*-nitrosomethylaniline was produced in mice injected intraperitoneally with methylaniline and gavaged with amyl nitrite; and (vi) nitrosating agents may occur normally in human skin lipids.

We showed previously that exposure of mice to nitrogen dioxide produces nitrosating agents in skin lipids, which react *in vitro* with amines to produce *N*-nitrosamines (Mirvish *et al.*, 1983), and that nitrogen dioxide bubbled into hexane solutions of methyl linoleate produces nitrosating agents (Mirvish & Sams, 1984). Since such agents could be significant for *N*-nitroso compound formation *in vivo* and in chemical and food systems, these findings have been pursued.

(i) *N*-Nitrosomethylaniline (NMA) formation in skin of mice exposed to nitrogen dioxide

To test whether nitrosating agents can produce nitrosamines *in vivo*, mice were exposed to 50 ppm nitrogen dioxide for 4 h, kept for 20 h, painted on the skin with 25 mg morpholine/mouse, left 0-1 h and killed. The *N*-nitrosomorpholine (NMOR) yield in the skin was  $0.28 \pm 0.28$  (18) nmol/mouse [mean  $\pm$  SD (no. of expts)]. Control experiments indicated that this yield was not significant. In similar experiments, 25 mg *N*-methylaniline were painted, and  $18 \pm 7$  (8),  $5 \pm 5$  (11) and  $0.15 \pm 0.29$  (6) nmol NMA/mouse were measured after 40, 70 and 130 min, respectively. Sulfamate, ascorbate and  $\alpha$ -tocopherol were included in the workup to prevent artefactual nitrosation. At 50 min, NMA yield in the hair was 87% of that in total skin. (These mice were shaved after they were killed.) The hair also contained 6.5% by weight of skin lipids and 47% of skin nitrosating agent. Nitrosating agent and NMA contents were similar in hair (6.1-6.2 nmol/mouse), but in shaved skin the NMA yield was only 13% of the nitrosating agent content.

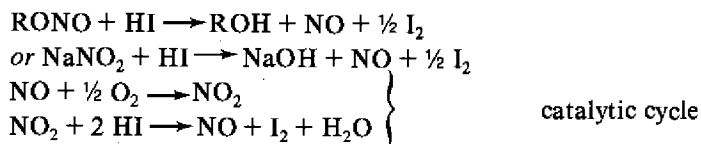
(ii) *Cholesterol as a precursor of nitrosating agent in mouse skin*

In a published study (Mirvish *et al.*, 1986), cholesterol was identified as a principal precursor of nitrosating agent in mouse skin lipids, yielding 60% of nitrosating agents produced when these lipids were reacted with nitrogen dioxide. Another 30% of the precursor was associated with the triglyceride fraction. Bubbling of nitrogen dioxide into cholesterol solutions in hexane gave 13% yields of a nitrosating agent, identified as cholesteryl nitrite. We obtained strong indications that the main skin nitrosating agent was cholesteryl nitrite.

(iii) *Enhancement of lipid and iodide autoxidation by cholesteryl nitrite*

Nitrite esters readily yield alkoxy radicals on heating or exposure to light, according to the reaction:  $\text{RONO} \rightarrow \text{RO} \cdot + \text{NO}$  (Smith, 1965). Hence, the presence of cholesteryl nitrite in skin could produce radical-catalysed lipid peroxidation, a process that has been associated with tumour promotion (Cerutti, 1985). To test for this activity *in vitro*, 1.0 g methyl linoleate or mouse skin lipids, with and without 50 mg cholesteryl nitrite, were stored in air with normal lighting for seven days. The product was analysed for conjugated diene by ultra-violet absorption at 233 nm, for hydroperoxide by iodometry, and for materials that react with thiobarbituric acid (Buege & Aust, 1978). The addition of cholesteryl nitrite increased autoxidation, measured by assays for conjugated diene and thiobarbituric acid-reacting material, by factors of 5-19. In an *in-vivo* experiment, thiobarbituric acid values for mouse skin lipids were increased from the normal 0.4 to 4-5 nmol/mg lipid one to two days after 12.5 mg cholesteryl nitrite/mouse were painted on the skin. Hence, cholesteryl nitrite promoted peroxidation of these lipids.

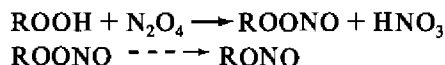
The iodometric assay gave extremely high values. This assay involves reaction with potassium iodide in 1 ml glacial acetic acid:chloroform (3:2) in an 8-ml screw-capped tube, followed by reaction with cadmium acetate and colorimetry at 353 nm. Further study showed that both cholesteryl nitrite and sodium nitrite catalysed the oxidation of 30-50 mol iodide/mol catalyst. When 110 ppm nitrogen dioxide in air (but not in nitrogen) were bubbled into the potassium iodide solution for 30 sec and the tube was then capped, this also caused rapid oxidation to iodine. We attribute the catalysis to the sequence shown below. Similar reactions of nitrite and of nitrogen dioxide have been reported before (Durrant & Durrant, 1970). When nitrogen was bubbled in during colour development, sodium nitrite and cholesteryl nitrite gave little colour, but cumene hydroperoxide produced 64% of the normal colour. This condition could be used to determine hydroperoxide in the presence of nitrite ester.

(iv) *Nitrosating agent precursor in peroxide fraction of autoxidized methyl linoleate*

As indicated in section (ii), 30% of nitrogen dioxide-derived skin nitrosating agent was associated with the triglyceride fraction. To study this agent, we bubbled nitrogen dioxide into methyl linoleate solutions in hexane. The nitrosating agent and its precursor were associated with the fraction that travelled on thin-layer chromatography (TLC, silica gel, hexane:ethyl acetate, 9:1) more slowly than methyl linoleate (Mirvish & Sams, 1984, and

unpublished results). Analysis of the TLC fractions by our three measures of lipid peroxidation showed that the band of nitrosating agent precursor also contained the peroxidation products.

When methyl linoleate was air-oxidized for up to seven days, it showed steady increases in the three peroxidation measurements and a two- to three-fold increase in nitrosating agent precursor. Samples of the oxidized methyl linoleate were subjected to TLC as before. Fractions at Rf 0.4-0.5 and 0.5-0.8 contained significant material by weight. The lower Rf fraction contained almost all the peroxidized material and 50% of the nitrosating agent precursor. More than twice as much nitrosating precursor was recovered from the TLC plate as was applied, indicating that precursor was produced on the plate. The nitrosating agents could be nitrite esters derived from peroxy nitrite esters formed from hydroperoxides (Pryor *et al.*, 1985):



(v) *In-vivo formation of NMA from amyl nitrite*

The finding that cholesteryl nitrite can produce nitrosamines (Mirvish *et al.*, 1986) and previous studies on nitrite esters (e.g., Loeppky *et al.*, 1984) suggest that amyl nitrite and butyl nitrite could produce nitrosamines *in vivo*. Newell *et al.* (1985) suggested that butyl nitrite, inhaled as a drug of abuse by homosexuals, could be involved in the etiology of acquired immune deficiency syndrome (AIDS). Patients with AIDS were more likely to develop Kaposi's sarcoma if they were exposed to butyl nitrite (Haverkos *et al.*, 1985), and hence this tumour could be induced by *N*-nitroso compounds formed from butyl nitrite. As a first step in studying this possibility, *N*-methylaniline (250 mg/kg) in water adjusted to pH 3 with hydrochloric acid (63 mg amine/ml) was injected intraperitoneally into adult male Swiss mice. After 30 min, amyl nitrite (40 mg/4 ml corn oil/kg) was given by gavage. After another 30 or 60 min, the mice were killed and the whole mice were analysed for NMA, as described in section (i). The NMA yield was 480 ± 310 (six mice) and 380 ± 100 (six mice) nmol NMA/mouse for animals killed after 30 and 60 min, respectively. When amyl nitrite was injected intraperitoneally and methylaniline was given by gavage, and the mice were killed 30 min later, the NMA yield was only 6 ± 11 nmol/mouse (four mice). In similar experiments in which amyl nitrite was given by gavage and mice were killed after 30 min, individual tissues contained 630 ± 280 (4, stomach contents), 90 ± 40 (3, stomach wall), 9 ± 4 (4, liver), 5 ± 6 (4, intestines with contents), 0.5 ± 0.7 (3, blood) and 13 (1, remaining tissue) nmol/g tissue (no. in parentheses, no. of analyses). Hence, most NMA was produced at the site of amyl nitrite administration. We will next study whether inhaled butyl nitrite can act similarly.

(vi) *Test for nitrosating agents in human skin lipids*

Human skin lipids might contain nitrosating agents, which could produce nitrosamines. Therefore, we analysed lipids from the faces of volunteers. Cotton-gauze pads wetted with acetone were rubbed over the faces. Acetone washings of the pads were evaporated, weighed and reacted with 10 mg morpholine/25 ml dichloromethane, as described by Mirvish *et al.* (1983). The product was concentrated and analysed for NMOR by GC-TEA. Thirteen lipid samples weighing 6-23 mg yielded 0.33 ± 0.26 nmol NMOR/sample, and eight pads without skin contact yielded 0.16 ± 0.13 nmol NMOR/sample (0.05 < *p* < 0.10). Six samples gave higher NMOR yields than the highest blank value. These results are inconclusive.

**Conclusions**

The results indicate that significant quantities of *N*-nitroso compounds might be produced in humans (i) on the skin from nitrosating agents produced by exposure to nitrogen dioxide and (ii) in internal organs from nitrite esters inhaled as vasodilators or drugs of abuse.

**Acknowledgements**

This work was supported by USPHS grants RO1-CA-32192, 1-S10-RR-01968 and CA-36727.

# NITRATE AS A PRECURSOR OF THE IN-VIVO FORMATION OF *N*-NITROSOMORPHOLINE IN THE STOMACH OF GUINEA-PIGS

M.O. García Roché<sup>1</sup> & D. Ziebarth<sup>2</sup>

<sup>1</sup>*Institute of Nutrition and Food Hygiene, Infanta 1158, City of Havana, Cuba;* and <sup>2</sup>*Central Institute of Cancer Research, Berlin-Buch, German Democratic Republic*

**Reduction of nitrates to nitrites and formation of the carcinogen *N*-nitrosomorpholine (NMOR) was investigated in the stomach of guinea-pigs. A semisynthetic diet with nitrate plus morpholine was administered intragastrically after a 24-h fast; after treatment, the animals were killed and stomach nitrite contents were determined 6, 12, 18, 24 and 30 min after the treatment using a colorimetric method. NMOR content was determined 18 min after treatment with nitrate plus morpholine using gas chromatography-thermal energy analysis. Reduction of nitrates to nitrites in the stomach was observed that was sufficient to synthesize NMOR in guinea-pigs under the conditions of this experiment.**

Formation of *N*-nitroso compounds in the gastrointestinal tract *in vivo* is probably the main source of human exposure (Reed, 1986). In the present paper, we report on endogenous formation of NMOR in the stomach of guinea-pigs after the administration of nitrate and morpholine.

In-vivo formation of carcinogenic *N*-nitroso compounds was first indicated by Sander and Bürkle (1969), and has subsequently been confirmed by many researchers. In experiments with animals, morpholine is the nitrosatable precursor of choice because it reacts readily with secondary amines of weak basicity (Mirvish, 1977) and because the carcinogenicity of NMOR has been well established (IARC, 1978a). In-vivo formation of NMOR, using morpholine and nitrite or sodium nitrite as precursors, has been demonstrated in rats (Mirvish *et al.*, 1981; Hecht & Morrison, 1984). We have used guinea-pigs because their gastric pH is closer to that of humans (Rickard & Dorough, 1984).

Many studies have shown nitrosation *in vivo* using nitrite as the precursor, but use of nitrate has received little attention. The daily intake of nitrite in some Cuban population groups has been estimated to be very low (1.04-2.4 mg/person per day; García Roché *et al.*, 1983, 1985, 1986), and therefore, in Cuba, nitrate intake could play a more important role in the carcinogenic risk of *N*-nitroso compounds. In order to evaluate the risk of nitrate ingestion, we first estimated nitrate consumption in Havana (65.9-276.8 mg/person per day; unpublished data). Secondly, we are carrying out experiments in animals to study in-vivo formation of *N*-nitroso compounds; the present paper reports on one of them.

## **In-vivo reduction of nitrate to nitrite**

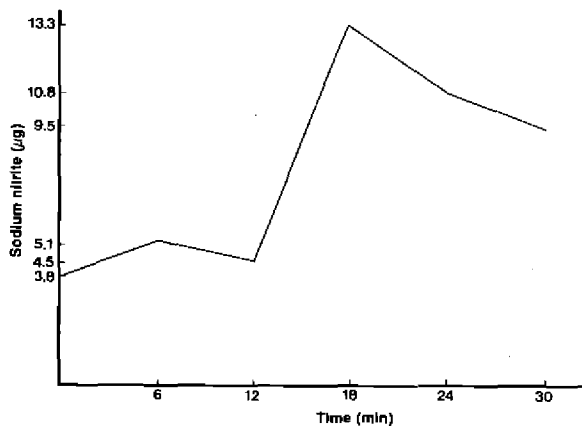
Five groups of three guinea-pigs were fasted for 24 h before treatment. Then, 200 mg of a semisynthetic diet (Table 1) plus 250 mg of sodium nitrate dissolved in 2 ml water were administered intragastrically. The synthetic diet contained no nitrate or nitrite. After

treatment, the animals were killed, and stomach nitrite was determined 6, 12, 18, 24 and 30 min later, using the sulfanilamide- $N_1$ -naphthylethylenediamine Cl colorimetric method. The largest amounts of sodium nitrite (12.7-13.9  $\mu\text{g}$ ) were found 18 min after administration of sodium nitrate (Fig. 1).

**Table 1. Composition of semi-synthetic diet**

Component	g/100 g dry weight
Casein	18
Sunflower-seed oil	8
Mineral mixture	5
Vitamin mixture	1
Cellulose	5
Starch	63

**Fig. 1. Nitrite content of guinea-pig stomach after administration of sodium nitrate**



### In-vivo formation of NMOR

After similar treatment, but including 250 mg morpholine, the NMOR content of the stomach was determined using a method that includes steam distillation, extraction with dichloromethane, concentration in a Kuderna-Danish and determination by gas chromatography coupled with thermal energy analysis, with the following conditions:

Equipment:	Chromatron Typ GCHF 18.3
Steel column:	2 m long and 3 mm i.d.
Column content:	Carbowax 20 M/terephthalic acid on Chromosorb WHP 80- to 100-mesh
Gas carrier:	Nitrogen, 3 l/h
Column temperature:	170°C
Injector temperature:	180°C
Detector:	Thermo Electron TEA 502-A
Pyrolysis temperature:	480°C
Cooled with liquid nitrogen	
Oxygen stream:	125 ml/min

The results are shown in Table 2.

In-vivo formation of the carcinogen NMOR using sodium nitrate as precursor has not often been investigated. It is a useful model, since it shows the real possibility of endogenous nitrosation after nitrate intake, a contaminant and additive always present in foodstuffs at relatively high concentrations.

**Table 2. In-vivo formation of NMOR in the stomach of guinea-pigs**

Stomach pH	Treatment	NMOR	
		$\mu\text{g}$	$\mu\text{g}/\text{kg}$
2.6-2.8	Control	0	0
2.5-2.9	250 mg sodium nitrate	0	0
2.9-3.0	250 mg morpholine	0 - 0.17	7.7
2.6-3.2	250 mg sodium nitrate + 250 mg morpholine	1.1-3.16	110-395

**Acknowledgements**

We thank Dr A. Castillo, Dr T. González, Dr M. Abreu, Mrs A.M. García and Miss I. Vega for their collaboration in this work.

# ROLES OF CYSTEINE AS BOTH PRECURSOR OF THIAZOLIDINE 4-CARBOXYLIC ACIDS FOUND IN HUMAN URINE AND POSSIBLE INHIBITOR OF ENDOGENOUS *N*-NITROSATION

I.K. O'Neill & H. Ohshima

*International Agency for Research on Cancer,  
150 cours Albert Thomas, 69372 Lyon, France*

In order to compare the utility and significance of 2-R-*N*-nitrosothiazolidine 4-carboxylic acids excreted in human urine as an index for exposure to *N*-nitroso compounds, the differences in formation of *N*-nitrosothiazolidine 4-carboxylic acid (NTCA; R=H) and *N*-nitroso-2-methylthiazolidine 4-carboxylic acid (NMTCA; R=CH<sub>3</sub>) were studied *in vitro*. It was determined that NMTCA has a 3:1 *trans:cis* stereoisomer ratio, while NTCA has a 1:1 *trans:cis* ratio; nitrosation acts on a pH-dependent equilibrium mixture of cysteine and aldehyde  $\rightleftharpoons$  thiazolidine 4-carboxylic acid, with cysteine blocking *N*-nitrosation. Previous reports on 2-R-*N*-nitrosothiazolidine 4-carboxylic acids in human urine show widespread involvement of cysteine, which has a dual role with nitrosating species. In view of this and the rapid blocking of *N*-nitrosation and slow *trans*-nitrosation by cysteine at acid pH, it is suggested that there may be a hitherto unrecognized protective role of thiol functions in dietary constituents.

It has been shown (Ohshima *et al.*, 1983; Tsuda *et al.*, 1983) that *N*-nitrosothiazolidine 4-carboxylic acids (NTCA and NMTCA) are excreted in human urine, NMTCA being an unassigned mixture of *cis:trans* stereoisomers. It was suggested that they could be formed partly through two endogenous reactions from cysteine and aldehyde, yielding precursor thiazolidines followed by nitrosation (Ohshima *et al.*, 1984b). Since cysteine and protein-bound cysteine are ubiquitous components of the human diet, the occurrence of *N*-nitrosating species and aldehydes in food and *in vivo* must lead to particularly common processes of formation of *N*-nitrosothiazolidine 4-carboxylic acids and/or *S*-nitrosation of cysteine. These reactions may be relevant both to the eventual appearance of NTCA/-NMTCA in human urine as indices of *N*-nitrosation and to findings of some experimental (Mirvish *et al.*, 1980) and epidemiological studies on gastrointestinal-tract cancers (Haenszel & Correa, 1975; Ziegler *et al.*, 1981; Tuyns *et al.*, 1985), which have detected either a strong or inferrable protective effect related to the source of dietary protein. The present work examines NTCA and NMTCA formation *in vitro* as indicators of endogenous *N*-nitrosation and the conflicting role of cysteine in this regard.

## Stereochemistry and formation of NTCA and NMTCA

The proton nuclear magnetic resonance (<sup>1</sup>H-NMR) spectra of synthetic NTCA (R=H) and NMTCA (R=CH<sub>3</sub>) were consistent with the anticipated presence of *E/Z* *N*-nitroso rotamers in NTCA and NMTCA, with the added complication of *cis:trans* 2-methyl isomers in NTCA. Methylated NTCA and NMTCA, either extracted from human urine or



synthesized, were always eluted in gas chromatographic-thermal energy analysis (GC-TEA) as one peak and as a three-to-one pair of peaks, respectively. It was of interest to define NMTCA stereochemistry, since the precursor methylthiazolidine 4-carboxylic acid (MTCA) has an approximately one-to-one pair of *cis:trans* isomers, and the substantially different isomer ratio of the *N*-nitroso derivatives might provide insight into MTCA formation and its *N*-nitrosation *in vivo*.

Partial separation of the NMTCA mixture by column chromatography prior to <sup>1</sup>H-NMR spectroscopic examination allowed distinction of *E* and *Z* pairs of NMTCA, but did not permit unambiguous assignment of *cis:trans* stereochemistry. Two fundamental steric aspects of cyclic *N*-nitrosamino acids were considered: (i) the cyclic *N*-nitrosamine group has a strong steric orientating influence on neighbouring substituents, and (ii) the methyl ester of NMTCA has the same *E:Z* rotamer ratio as NMTCA (Table 1), thus excluding favourable hydrogen-bonding between the *N*-nitroso and carboxylate functions. The data indicate that the *N*-nitroso group has a largely dipolar character that requires an essentially planar arrangement for all five of the N=NO and attached C atoms (Scheme 1) and with a high energy barrier for rotamer interconversion. The analogous *N*-nitroso(2-ethylthiazolidine) 4-carboxylic acids (NETCA) were found to have a similar isomer/rotamer distribution by <sup>1</sup>H-NMR (Table 1) and by GC-TEA as NMTCA. However, the NMR spectra showed restricted rotation arising from the extra bulk of the 2-ethyl function (Scheme 1), allowing the major isomer to be assigned as *trans*; thus, the NMTCA isomer with shorter retention time in GC chromatography is *trans* by analogy.

**Table 1. Proportions of *Z/E* rotamers in *N*-nitroso-thiazolidines**

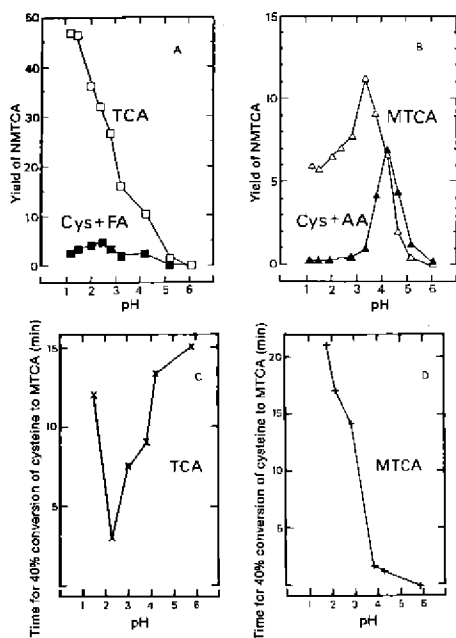
Compound <sup>a</sup>	R <sub>2</sub>	R <sub>4</sub>	<i>Z/E</i>
NTCA	H	CO <sub>2</sub> H	50/50
NMTCA	CH <sub>3</sub> ( <i>cis</i> )	CO <sub>2</sub> H	84/16
Me-NMTCA	CH <sub>3</sub> ( <i>cis</i> )	CO <sub>2</sub> CH <sub>3</sub>	86/14
NETCA	C <sub>2</sub> H <sub>5</sub> ( <i>cis</i> )	CO <sub>2</sub> H	85/15
NMTCA	CH <sub>3</sub> ( <i>trans</i> )	CO <sub>2</sub> H	72/28
NETCA	C <sub>2</sub> H <sub>5</sub> ( <i>trans</i> )	CO <sub>2</sub> H	82/18

<sup>a</sup>Me-NMTCA, methyl ester of NMTCA; NETCA, ethyl ester of NMTCA; estimated ± 3%; measured by 350 MHz <sup>1</sup>H-NMR in acetone-D<sub>6</sub> at 20°C

direct observation of reaction mixtures, simultaneous detection of all organic components and identification of stereoisomers. It was found that there is a difference in the pH-dependency of formation of TCA and MTCA from cysteine with formaldehyde and acetaldehyde (Fig. 1C,D). Thus, differences in formation of NTCA and NMTCA from nitrosation of cysteine-aldehyde mixtures must arise in part from the pH-dependent nature of the equilibria between cysteine-aldehyde and the TCA/MTCA product.

In previous work (Ohshima *et al.*, 1984b), it was shown that thiazolidine 4-carboxylic acid (TCA) and MTCA had considerably different pH profiles and yields in *N*-nitrosation (Fig. 1A,B) and that NTCA and NMTCA were formed *in vitro* and *in vivo* (Ohshima *et al.*, 1984a) in much greater yields by nitrosation of preformed TCA and MTCA than of mixtures of cysteine with either formaldehyde or acetaldehyde. High-field <sup>1</sup>H-NMR spectroscopy was utilized to observe formation of TCA and MTCA, as it allowed

**Fig. 1. pH profiles for formation *in vitro* after 30 min of NTCA (A), NMTCA (B), TCA (C) and MTCA (D)**



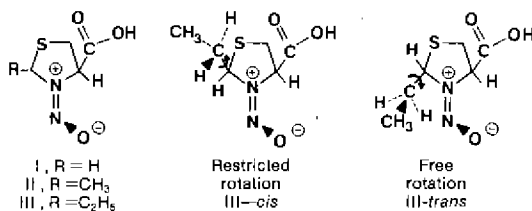
NTCA (□) nitrosation of TCA; NMTCA (Δ) nitrosation of MTCA; NTCA (■) nitrosation of L-cysteine and formaldehyde (FA); NMTCA (▲) nitrosation of L-cysteine and acetaldehyde (AA); TCA (×) 40% conversion of cysteine (30 mM) with FA (30 mM); MTCA (+) 10% conversion of cysteine (30 mM) with AA (30 mM). Conditions for NTCA and NMTCA formation given by Ohshima *et al.* (1984)

and NMTCA in that *cis*-MTCA is *N*-nitrosated only slowly to yield sterically compressed *cis*-NMTCA. They also indicate that *N*-nitrosation of preformed MTCA cannot be as reliable a guide for endogenous *N*-nitrosation conditions as formation of NTCA, this being made even more unfavourable by the slower rate of cysteine-acetaldehyde reaction and the much slower production of NMTCA than NTCA from the simultaneous presence of nitrosating species, cysteine and aldehyde.

#### Effect of presence of cysteine during nitrosation of MTCA or morpholine

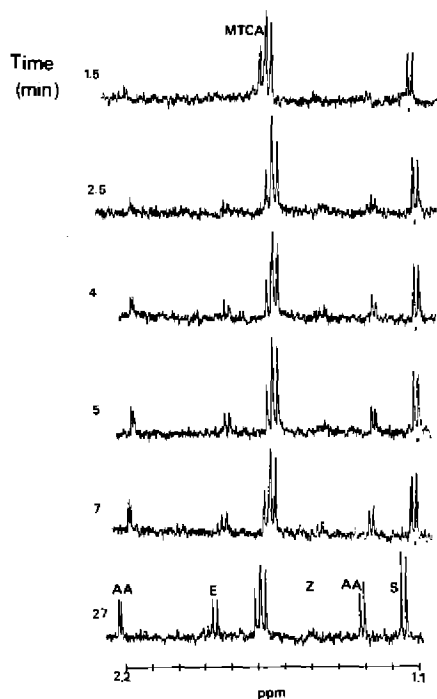
It is known that cysteine and other thiols are very rapidly *S*-nitrosated by *N*-nitrosating species. Davies *et al.* (1978) have shown that *S*-nitroso cysteine can undertake transnitrosation to yield *N*-nitroso compounds, but at a much slower rate and giving lower yields than direct *N*-nitrosation. The nitrosation of MTCA was performed at pH 4.7, conditions chosen to give relatively slower direct *N*-nitrosation (Fig. 1) but presumably satisfactory both for

#### Scheme 1



In order to observe the differential reactions of the *cis* and *trans* isomers by NMR spectroscopy, nitrosation of MTCA was performed with insufficient amounts of nitrite at pH 1.6 (Fig. 2) and found to give acetaldehyde and *S*-nitrosocysteine in similar overall yield to NMTCA. The minor MTCA isomer, *cis*-MTCA (Pesek, 1978), disappeared more rapidly and, when most of the nitrite had been consumed, the *cis:trans* ratio of the residual MTCA was quickly re-established, thus demonstrating the slow equilibration *via* the opening Schiff base. These results are consistent with the nitrosation of three interconverting MTCA components, namely, in order of rapidity, the open-ring Schiff base, *trans*-MTCA and *cis*-MTCA. Under the same pH conditions and in the 30-min duration of the above experiment, preformed NMTCA was not significantly decomposed and TCA was nitrosated and produced only NTCA. These observations together account for the difference in *cis:trans* isomer ratios of MTCA

**Fig. 2. Proton Fourier transform nuclear magnetic resonance spectra of methyl resonances of *cis:trans* MTCA and its products at various times after mixing at pD1.3 with 0.6 equivalents of sodium nitrite**



The *trans:cis* ratio is 1.26, 1.41, 1.63, 2.00, 1.84 and 1.48 in sequence 1.5 to 27 min. In the reaction mixture, AA designates acetaldehyde methyls; E the *trans:cis* E rotamers of NMTCA, Z the *trans:cis* Z rotamers of NMTCA, with S a non-reactive standard unaffected by the reaction.

was not detectable by NMR, and GC-TEA analysis confirmed it as being only slightly greater than that formed in the presence of ascorbic acid (Table 3). Cysteine caused no significant denitrosation of NMOR, and preformed *S*-nitrosocysteine gave no significant transnitrosation to morpholine within the short reaction time; this is consistent with the data of Davies *et al.* (1978).

*S*-nitrosation (Tu *et al.*, 1984) and transnitrosation (Davies *et al.*, 1978) by any *S*-nitrosothiol produced. In the absence of added cysteine, NMTCA was produced with a similar yield of the by-products acetaldehyde and *S*-nitrosocysteine (Table 2). In the presence of 1.5 equivalents of cysteine, no free acetaldehyde was observed but the yield of NMTCA was greatly reduced and the amount of *S*-nitrosocysteine formed was much greater than that of NMTCA. Thus, the presence of added cysteine did not facilitate NMTCA formation *via* partial reversal of the (cysteine/acetaldehyde  $\rightleftharpoons$  MTCA) equilibrium, but, instead, led to preferential formation of *S*-nitrosocysteine as an alternative product. Therefore, co-occurrence of cysteine and nitrosating species does not enhance the formation of NMTCA but reduces it, despite the relatively rapid *N*-nitrosation of MTCA compared to most amines (Ohshima *et al.*, 1984a). This is consistent with the rather low yields of both NTCA and NMTCA (Fig. 1A,B) from *N*-nitrosation of cysteine-aldehyde mixtures compared with that of preformed thiazolidine precursors.

Several sequences of *N*-nitrosation were conducted utilizing morpholine (to avoid the equilibria associated with the thiazolidines) in order to examine the effect of the relative times of appearance of cysteine and nitrosating species during *N*-nitrosation of amines (Table 3). Reagents were mixed and then observed by NMR spectroscopy after 2 and 10 min; the reaction was then allowed to proceed for a total of 30 min before quenching with acidified aqueous ammonium sulfamate prior to analysis by GC-TEA. With cysteine added before or at the same time as nitrosating agent, the amount of *N*-nitrosomorpholine (NMOR) formed within a short time

**Table 2. Effect on nitrosation of NMTCA of nitrite and cysteine as determined by  $^1\text{H-NMR}$  spectroscopy<sup>a</sup>**

Nitrite (mol/l)	Cysteine (mol/l)	Yield of NMTCA (%)	Yield of acetaldehyde (%)	Yield <sup>b</sup> of <i>S</i> -nitroso cysteine (%)
0.012	0	3.5	5.1	~ 6
0.036	0	14.5	12.5	~ 12
0.036	0.055	3.8	<1	~ 24
0.072	0.055	7.1	<1	~ 49

<sup>a</sup>Aqueous MTCA solution was mixed with a  $\text{D}_2\text{O}$  solution of sodium nitrite in the presence or absence of cysteine to give a final concentration of 0.033 M MTCA in 0.2 M potassium phosphate in  $\text{D}_2\text{O}$ , pD 4.7; 350 MHz  $^1\text{H-NMR}$  spectra taken 10 min after mixing to detect NMTCA methyls at 1.51, 1.88 and 1.91 ppm; acetaldehyde methyls at 1.32 and 2.2 ppm; *S*-nitrosocysteine as multiplets 4.0 to 4.2 ppm

<sup>b</sup> Relative to original MTCA

**Table 3. Effect on *N*-nitrosation of morpholine of presence or later addition of cysteine and other modifiers**

Reaction <sup>a</sup>	% Conversion of MOR to NMOR: time of NMR determination after nitrite addition		TEA analysis for NMOR yield relative to first reaction
	2 min	10 min	
MOR + $\text{NO}_2^-$	9.8	20.9	1.0
MOR + $\text{NO}_2^-$ , then cys at 2 min	19.2	20.4	1.0
MOR + cys	ND	ND	0.0002
MOR + cys, then $\text{NO}_2^-$ at 3 min	ND	ND	0.02
MOR + SMecys, then $\text{NO}_2^-$ at 3 min	7.5	15.8	1.0
MOR + ascorbate, then $\text{NO}_2^-$ at 3 min	ND	ND	0.006
MOR + $\text{SCN}^-$ , then $\text{NO}_2^-$ at 3 min	13.5	53	0.97
$\text{NO}_2^-$ + SMecys, then MOR at 3 min	2.4	9.2	0.87

<sup>a</sup>In the sequences stated, to 0.8 ml  $\text{DCl/KCl}$  buffer pD2 was added either cysteine (cys), *S*-methyleysteine (SMecys), sodium thiocyanate ( $\text{SCN}^-$ ) or sodium ascorbate (0.2 mmol in 0.5 ml  $\text{D}_2\text{O}$ ) together with morpholine hydrochloride (MOR; 0.2 mmol in 0.2 ml  $\text{D}_2\text{O}$ ) and sodium nitrite ( $\text{NO}_2^-$ ; 0.1 mmol in 0.1 ml  $\text{D}_2\text{O}$ ). After 30 min, excess acidified ammonium sulfamate was added to block further nitrosation. ND, not detected

### Significance for *in-vivo* nitrosation

The effect of cysteine in greatly reducing or abolishing *N*-nitrosation of these rather rapidly nitrosatable amines could be of significance for endogenous *N*-nitrosation, both in regard to monitoring the process and as a possible protective effect of cysteine

and thiols. Recommended daily intakes for adult males of the sulfur-containing amino acids are much greater (1100 mg; Food and Agriculture Organization/World Health Organization, 1973) than the US recommended intakes of vitamin C (45 mg) or vitamin E (15 mg). Neither Hirayama (1971) nor Correa *et al.* (1983) found vitamin C deficits in Japanese or Colombian populations at elevated risk for gastric cancer, although both groups have concluded that deficits of animal protein or milk are associated. Nitrite is known to *S*-nitrosate the thiols of meat protein (Byler *et al.*, 1983); fresh meat protein has free thiols at 21-25 mM levels (Hamm & Hoffmann, 1966), whereas nitrite in gastric juice occurs at low micromolar concentrations.

The present experiments show a complex system in which cysteine is both a precursor and an inhibitor of the formation of *N*-nitroso compounds, NMTCA being formed only in partial yields and thus unlikely to be suitable as a quantitative indicator for monitoring endogenous *N*-nitrosation. In support of this conclusion, Lu *et al.* (1986), studying inhabitants at high risk for oesophageal cancer in China who have a very low animal protein intake, have shown that urinary NTCA excretion is well correlated with other urinary indicators of endogenous nitrosation and nitrate excretion, whereas NMTCA is not. Also, the data of Tsuda *et al.* (1986) have shown that NTCA excretion is a more reliable indicator than NMTCA of endogenous nitrosation after exposure to tobacco smoke for a subject on a fixed diet. These studies further demonstrate the universality of cysteine involvement in endogenous *N*-nitrosation. More work is required to elucidate the possible role of cysteine as a factor in reducing endogenous *N*-nitrosation in the context of the possible protective effects of animal protein suggested by various epidemiological studies.

# MODEL RISK ANALYSIS OF NITROSATABLE COMPOUNDS IN THE DIET AS PRECURSORS OF POTENTIAL ENDOGENOUS CARCINOGENS

S.E. Shephard, C. Schlatter & W.K. Lutz

*Institute of Toxicology, Swiss Federal Institute of Technology,  
and University of Zurich, CH-8603 Schwerzenbach, Switzerland*

The potential health risk posed by the endogenous formation of *N*-nitroso compounds (NOC) from nitrosation of dietary ureas, guanidines, amides, amino acids and amines (primary, secondary and aromatic) was estimated according to the model:

$$\text{Risk} = [\text{daily intake of precursor}] \times [\text{gastric concentration of nitrite}]^n \times [\text{nitrosatability rate constant}] \times [\text{carcinogenicity of derivative}].$$

The daily intakes of these compound classes span five orders of magnitude (100 g/day amides, top; 1-10 mg/day secondary amines, ureas, bottom); the nitrosation rate constants span seven orders of magnitude (aryl amines, ureas, top; amides, secondary amines, bottom); and the carcinogenicity estimates span a 10 000-fold range from 'very strong' to 'virtually noncarcinogenic'. The resulting risk estimates likewise span an enormous range (nine orders of magnitude): dietary ureas and aromatic amines combined with high nitrite concentration could pose as great a risk as the intake of preformed *N*-nitrosodimethylamine in the diet. In contrast, the risk posed by the in-vivo nitrosation of primary and secondary amines is probably negligible. The risk contributed by amides (including protein), guanidines and primary amino acids is intermediate between these two extremes.

The human diet contains a variety of nitrosatable precursors, which differ markedly with respect to (i) daily intake, (ii) rate of nitrosation, and (iii) carcinogenicity of the nitroso derivative. After combination of all three variables, the different precursor classes were evaluated for their relative importance as a potential source of endogenous carcinogens (Shephard *et al.*, 1987).

## Precursors of *N*-nitroso compounds in the diet

The substances of interest in this model risk assessment were those nitrosatable compounds widely found in the natural human diet: amines, amino acids, amides, guanidines and ureas. Unlike industrial or pharmaceutical NOC precursors, these substances constitute an unavoidable source of nitrosatable substances for the general population. The average daily intake of each precursor, *C*, was then calculated as shown in Equation 1 on the basis of average eating habits in Europe, using 1980 food consumption statistics (Schweizerisches Bauernsekretariat, 1983).

$$\text{Daily intake}_C = \sum_i [\text{amount of } C \text{ in food item}_i] \times [\text{daily intake of food item}_i]. \quad (1)$$

Not surprisingly, those compounds of nutritional or biological importance, and their derivatives, are the precursors consumed in the largest amounts (Table 1). Amides are the most important precursor class in the diet: in the form of proteins, they are consumed at a level of almost 100 000 mg/day from meat, poultry, fish, milk, eggs, cheese and grain products (Schweizerisches Bauernsekretariat, 1983). Guanidines form the second most important group: creatine and creatinine are important constituents of meat, comprising about 2% of the total protein; daily consumption of guanidines amounts to 1000 mg. Free amino acids are also found in all protein-rich foods: glutamic acid, glycine and alanine predominate, and total daily intake amounts to 10-50 mg. Proline (found in collagen fibres) is the major secondary amino acid; intake of free proline is about 0.5 mg/day. Primary amines, formed by decarboxylation of amino acids during microbial fermentation of foods, are eaten at a level of 100 mg/day; cheese and preserved meats are the major sources of biogenic amines. The ureas citrulline and ornithine are also of biological importance, but analytical methods for their detection are lacking; a preliminary estimate of daily intake is  $> 1$  mg/day. Secondary amines and aryl amines are not nutritionally important classes; they are found in trace amounts in most foods, and daily intakes amount to approximately 5 and 2 mg, respectively.

### Nitrosation rate

The chemistry and kinetics of the nitrosation of amine- and amide-type compounds are well understood (reviewed by Mirvish, 1975). The most important factor governing the nitrosation rate of aliphatic and aromatic amines is the pK of the amine group (lower pK<sub>a</sub>  $\propto$  faster rate). The amino acids have the additional possibility of intramolecular catalysis by the carboxyl group, which increases their nitrosation rate approximately 100 fold over that of the simple amines. Resonance forms that donate electron density to the N atom determine the relative nitrosation speeds of the amide-type compounds: ureas  $>$  amides = guanidines.

Kinetic studies have been carried out on only a few of the compounds considered in this model risk assessment, and estimates for the primary amines and primary amino acids had to be based on data from their secondary analogues. Estimates of rate constants  $k_2$  for amines at optimal pH and  $k_6$  for amides at pH 2 (nomenclature from Mirvish, 1975) were made by comparison with the in-vitro values reported by Ridd (1961) and Mirvish (1975), using the above guidelines and the general chemical principle that bulky compounds sterically hinder a reaction. The individual estimates are given in Table 1. As classes, the precursors can be ranked as follows: aryl amines  $>$  ureas  $>$  primary amino acids  $>$  secondary amino acids  $>$  primary amines  $>$  secondary amines = guanidines  $>$  amides, with respect to ease of nitrosation. The estimates span seven orders of magnitude.

### In-vivo yield of N-nitroso compounds

The in-vitro rate constants at 25°C correspond reasonably well to in-vivo rates at 37°C (reviewed by Shephard *et al.*, 1987). The in-vivo yields of NOC were calculated assuming a stomach volume of 1 litre, a reaction time of 1 h, a pH optimal for the reaction to proceed (2.5-3.4 for the amines, and 2.0 for the amide-type compounds), and two realistic gastric nitrite concentrations taken from the literature: 'low nitrite' = 1.7  $\mu$ M (Klein *et al.*, 1978) and 'high nitrite' = 72  $\mu$ M (Tannenbaum *et al.*, 1974). At the low nitrite concentration, the most significant yields of NOC come from protein, 800 pmol, and from methylurea, 400 pmol, followed by the guanidines and aryl amines (50 pmol each). Because of greater sensitivity to nitrite concentration, the in-vivo nitrosation of aryl amines becomes even more

**Table 1. Estimates of health risks posed by gastric in-vivo nitrosation of food precursors relative to consumption of preformed NDMA**

Precursor	Daily intake (mg)	Rate constant estimate <sup>a</sup>	Carcinogenic potency (OPI)	Health risk relative to NDMA (risk from NDMA = 1)	
				[nitrite]=1.7 $\mu$ M	[nitrite]=72 $\mu$ M
<b>PRIMARY AMINES</b>					
Spermidine	35	0.005	100	$1 \times 10^{-7}$	$2 \times 10^{-4}$
Tyramine	21	0.05	100	$7 \times 10^{-7}$	$1 \times 10^{-3}$
Cadaverine	15	0.01	100	$1 \times 10^{-7}$	$3 \times 10^{-4}$
Putrescine	15	0.01	100	$2 \times 10^{-7}$	$3 \times 10^{-4}$
Methylamine	3	0.005	$10^3$	$4 \times 10^{-7}$	$8 \times 10^{-4}$
Total	100			$10^{-6}$	$10^{-3}$
<b>PRIMARY AMINO ACIDS</b>					
Glutamic acid	> 3.2	1	10	$1 \times 10^{-6}$	$2 \times 10^{-3}$
Glycine	> 1.3	1	100	$1 \times 10^{-5}$	$1 \times 10^{-2}$
Alanine	> 0.4	1	100	$3 \times 10^{-6}$	$8 \times 10^{-3}$
Total	> 10			$10^{-5}$	$10^{-2}$
<b>SECONDARY AMINES</b>					
Dimethylamine	1.7	0.002 <sup>b</sup>	$10^3$	$7 \times 10^{-8}$	$2 \times 10^{-4}$
<i>N</i> -Methylbenzylamine	0.6	0.013 <sup>b</sup>	$10^3$	$7 \times 10^{-7}$	$1 \times 10^{-3}$
Pyrrolidine	0.6	0.005 <sup>b</sup>	100	$3 \times 10^{-9}$	$8 \times 10^{-6}$
Total	5			< $10^{-6}$	$10^{-3}$
<b>SECONDARY AMINO ACIDS</b>					
Proline	> 0.5	0.037 <sup>b</sup>	< 0.1	< $1 \times 10^{-10}$	< $1 \times 10^{-7}$
Sarcosine	1	0.23 <sup>b</sup>	1	$3 \times 10^{-9}$	$5 \times 10^{-6}$
Total	> 1			$10^{-9}$	< $10^{-3}$
<b>ARYL AMINES</b>					
<i>N</i> -Methylaniline	1.6	250 <sup>b</sup>	100	$4 \times 10^{-4}$	0.8
Aniline	1	500 <sup>b</sup>	100	$6 \times 10^{-4}$	1
Total	2			$10^{-3}$	> 1
<b>AMIDES</b>					
Protein	92 000	0.001	10	$8 \times 10^{-4}$	$4 \times 10^{-2}$
Carnosine	2000	0.001	1	$2 \times 10^{-7}$	$1 \times 10^{-5}$
Total	$10^5$			< $10^{-3}$	$10^{-2}$
<b>GUANIDINES</b>					
Creatine	800	0.004	1	$6 \times 10^{-6}$	$2 \times 10^{-4}$
Creatinine	300	0.004	10	$2 \times 10^{-5}$	$1 \times 10^{-3}$
Methyl guanidine	0.2	0.004 <sup>b</sup>	$10^3$	$1 \times 10^{-5}$	$6 \times 10^{-4}$
Total	$10^3$			< $10^{-4}$	< $10^{-2}$
<b>UREAS</b>					
Methyl urea	> 1	10.5 <sup>b</sup>	$10^3$	> $5 \times 10^{-2}$	> 1
<i>N</i> -Carbamoyl putrescine	1 <sup>c</sup>	1	100	$2 \times 10^{-5}$	$1 \times 10^{-3}$
Citrulline	1 <sup>c</sup>	0.7 <sup>b</sup>	10	$1 \times 10^{-5}$	$4 \times 10^{-4}$
Total	> 1			> $5 \times 10^{-2}$	> 1

<sup>a</sup> $k_2$  at optimal pH for amines;  $k_6$  at pH 2 for amide-type compounds<sup>b</sup>Rate constants from Mirvish (1975)<sup>c</sup>Preliminary estimate of 1 mg/day assigned



important than that of ureas or protein after a nitrite-rich meal (100 nmol *versus* 30 nmol). The amounts of nitrosamine produced *in vivo* from aliphatic amines are, in contrast, very small, comprising only picomole quantities even in the presence of large amounts of nitrite. The yields of *N*-nitrosamino acids lie between these two extremes, ranging from < 1 pmol at low nitrite concentrations to > 400 pmol at high nitrite levels.

#### Carcinogenicity of nitroso derivatives

The carcinogenic potency of the nitroso derivatives of each compound listed in Table 1 was estimated. The results of chronic feeding studies in rats were used as the data base, and the data were normalized using the Oncogenic Potency Index (OPI, adapted from Meselson & Russell, 1977):

$$\text{OPI} = \ln(1 - \text{tumour incidence}) / \text{daily dose} / (\text{time})^3. \quad (2)$$

Quite precise estimates of the OPI could be made for the secondary amines, secondary amino acids and ureas. No study was available on nitrosopeptides, the nitrosoguanidines of interest, or the unstable primary nitrosamine classes. Rough OPI estimates were made for these latter compound classes, using the following empirical guidelines (Druckrey *et al.*, 1967):

- (i) The short-chain alkyl substituted NOC are very potent: OPI 10<sup>3</sup>.
- (ii) The larger NOC become less potent: OPI 10-100.
- (iii) A stable compound with a polar or charged substituent is a weak or noncarcinogen: OPI < 0.1-1.

The potency of the unstable NOC depends on their half-life within the cell. *para*-Hydroxymethylbenzene diazonium ion, given orally to mice, and *N*-nitrosomethylamine, generated *in situ* in rat stomach, both produced covalently bound DNA adducts (Huber & Lutz, 1984; Shephard, Fischer & Lutz, in preparation). On the basis of this information, conservative potency estimates were made for the primary nitrosamines, assuming long enough lifetimes to reach the DNA.

#### Calculation of health risk

The health risk posed by *in-vivo* nitrosation of food components was compared to that posed by the presence of preformed *N*-nitrosodimethylamine (NDMA) in foods. Estimates of the health risks due to particular NOC were calculated using equation 3:

$$\begin{aligned} \text{Risk}_{\text{NOC}} = & \text{daily intake of precursor } C \text{ (mol/day)} \\ & \times \text{gastric concentration of nitrite}^n \text{ (1.7 or } 72 \times 10^{-6} \text{ M)}^n \\ & \times \text{nitrosatability rate constant } k_2 \text{ (s}^{-1}\text{M}^{-2}) \text{ or } k_6 \text{ (s}^{-1}\text{M}^{-2}) \\ & \times \text{carcinogenicity of derivative OPI (kg mmol}^{-1}\text{ day}^{-1}\text{ year}^{-3}). \end{aligned} \quad (3)$$

The parameter *n* is 2 for amines and 1 for amide-type precursors. The model assumes that health risk is linearly related to both the carcinogenicity and to the daily endogenous yield of each NOC. Similarly, the risk due to preformed NDMA (intake 10 nmol/day, Spiegelhalter *et al.*, 1980b; OPI value 3000, Parodi *et al.*, 1982) is 10 nmol × 3000 = 30 000. The relative risk can be expressed as Risk<sub>NOC</sub>/Risk<sub>NDMA</sub>.

Results of these calculations can also be found in Table 1. The risk estimate totals of the various precursor classes span a range of nine orders of magnitude. According to these calculations, the aryl amines and ureas are the most important precursor classes. Under conditions of high gastric nitrite concentration, endogenously formed *N*-nitrosoureas and aromatic nitrosamines could pose a risk equal to or greater than that of unavoidable NDMA in the diet. Guanidines, amides and primary amino acids fall into a medium-risk category. Under normal conditions, they constitute a risk of 0.1-1% that of NDMA, but under extreme conditions of high nitrite or low pH (guanidines and amides), they could also become important. The primary amines, secondary amines and secondary amino acids fall into the lowest class, and the risk posed by their nitrosation is negligible, which agrees with the conclusions reached by Fine *et al.* (1982).

Two priorities for future investigation emerge from this model risk analysis. Firstly, the sources and levels of arylamines and ureas in the diet should be studied comprehensively. This would allow a more realistic estimate of the *total* risk contributed by aryl amines and ureas. Secondly, the carcinogenic potencies of key nitrosated products should be determined more precisely than the necessarily vague categories presented here. Unfortunately, the instability of some *N*-nitroso derivatives precludes their testing in long-term studies. Work is currently in progress in our laboratory to develop short-term tests (Shephard *et al.*, this volume) that will allow us to characterize the overall reactivity (nitrosatability of precursor and alkylating power) or genotoxicity of dietary components that form unstable NOC.

**LABORATORY STUDIES ON FORMATION**

## FURTHER STUDIES ON MURINE MACROPHAGE SYNTHESIS OF NITRITE AND NITRATE

D.J. Stuehr & M.A. Marletta<sup>1</sup>

*Department of Applied Biological Sciences,  
Massachusetts Institute of Technology, Cambridge, MA 02139, USA*

Further studies on macrophage synthesis of nitrite and nitrate showed lipopolysaccharide (LPS) and interferon (IFN) to be potent stimuli. Kinetic experiments showed a time lag of 6 h for LPS and 10-12 h for IFN. The protein synthesis inhibitor cycloheximide completely inhibited nitrite and nitrate synthesis when present in the media at time 0 but had no effect if added at times after the lag period. A number of experiments were carried out to test the involvement of reactive oxygen species (the 'oxygen burst') in stimulated macrophage synthesis. All results were consistent with a lack of involvement of the oxygen burst in this process.

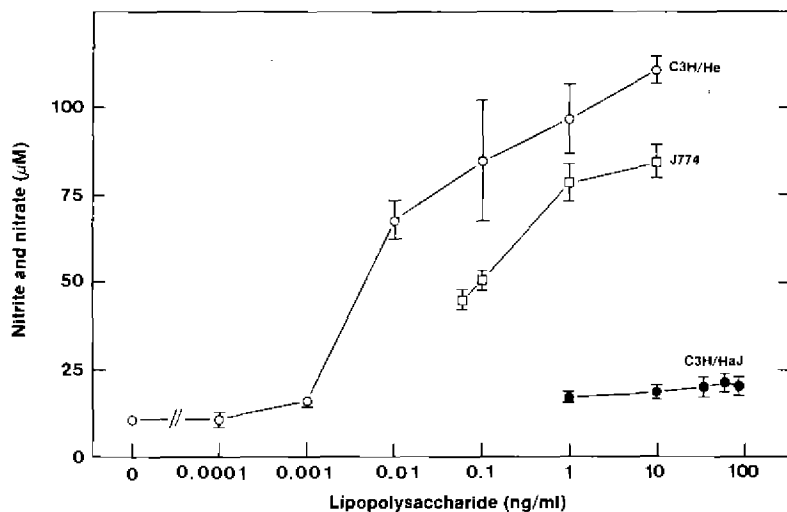
Subsequent to our finding that macrophages stimulated with *Escherichia coli* lipopolysaccharide (LPS) synthesize nitrite and nitrate (Stuehr & Marletta, 1985), we have investigated this synthesis using other stimuli and immortalized macrophage cell lines. The synthesis represents a novel metabolic route for mammals, and this, coupled with our finding that stimulated macrophages carry out *N*-nitrosations (Miwa *et al.*, this volume), led us to investigate the regulation and biochemistry of the process.

### Dose-response of macrophages to LPS and $\gamma$ -IFN

Figure 1 illustrates typical results obtained with macrophage cultures treated with LPS. Macrophages were isolated and plated at  $1 \times 10^6$  cells per well and were cultured in 1 ml of media, as described previously (Stuehr & Marletta, 1985). C3H/He mice are LPS responsive, and C3H/HeJ mice are a mutant strain that are resistant to the in-vivo effects of LPS. J774 cells are a murine-immortalized cell line which is responsive to LPS. We had previously observed that intraperitoneal injection of LPS led to about a five-fold increase in urinary nitrate levels in C3H/He mice but not in C3H/HeJ mice (Stuehr & Marletta, 1985). The results of other experiments reported in that paper led to the conclusion that it was the macrophage that was responsible for this immunostimulated synthesis. As expected, cells isolated from the C3H/He strain showed a dose-response curve, whereas cells from the C3H/HeJ strain did not synthesize nitrite or nitrate at any LPS concentration. The  $\gamma$ -IFN dose-response results with macrophages isolated from C3H/He and C3H/HeJ mice are shown in Figure 2. The result obtained with the J774 cell line led us to test LPS and  $\gamma$ -IFN in a number of other cell lines in order to find the most appropriate one(s) for studies of precursor/product studies, and this work is in progress. Additionally, the ability to respond or not might provide some information regarding the biochemistry of the process.

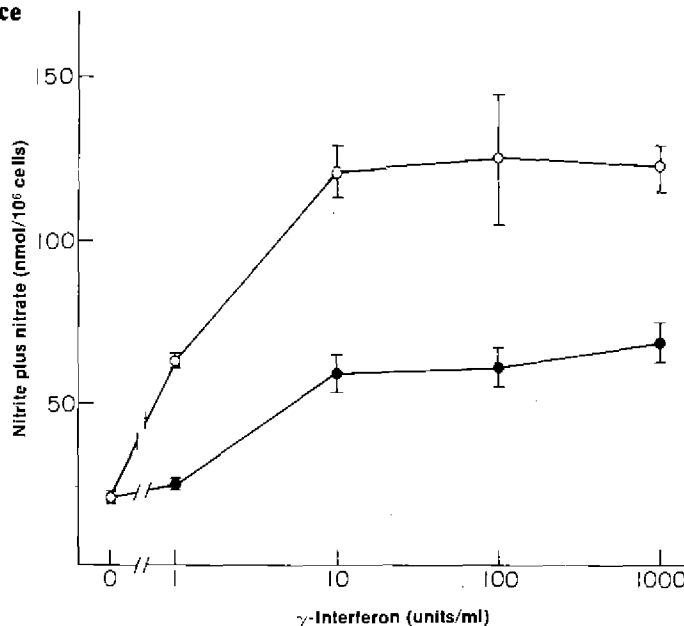
<sup>1</sup> To whom correspondence should be addressed

**Fig. 1. Dose-response with LPS as the stimulant of macrophages from C3H/He (○), J774 (□) and C3H/HeJ (●) mice**



Macrophages ( $1 \times 10^6$ /well) were cultured in 1 ml media containing various concentrations of LPS for 72 h. Each point represents the nitrite/nitrate concentration present at the end of the experiment, averaged for three wells. Nitrate and nitrite were analysed as described by Green *et al.* (1982).

**Fig. 2. Dose-response with  $\gamma$ -IFN as the stimulant of macrophages from C3H/He (○) and C3H/HeJ (●) mice**

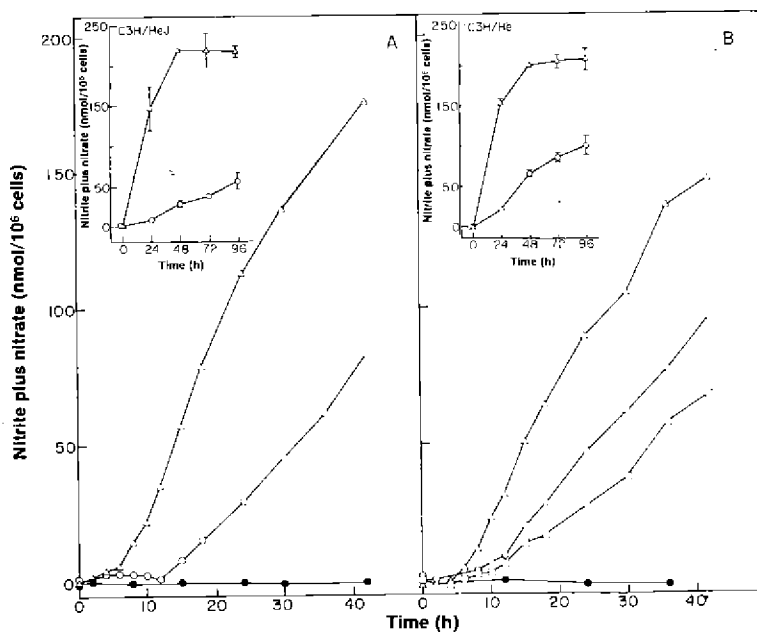


Macrophages ( $1 \times 10^6$  cells/well) were cultured for 72 h in 1 ml media containing various concentrations of  $\gamma$ -IFN. The nitrite/nitrate values are the concentrations in the supernatant at the end of the experiment.

### Kinetics of nitrite/nitrate synthesis

The kinetics of nitrite/nitrate synthesis in C3H/He and C3H/HeJ macrophages are shown in Figure 3. It can be seen that there is a lag before nitrite/nitrate synthesis begins, which is about 6 h with LPS as the stimulant and about 10-12 h with  $\gamma$ -IFN. These results give total nitrite and nitrate. If separate analyses are carried out, regardless of the stimulant, the ratio of nitrite to nitrate is 3:2 at all time points once synthesis begins.

**Fig. 3. Kinetics of nitrite/nitrate synthesis**



C3H/HeJ (panel A) and C3H/He (panel B) macrophages ( $1 \times 10^6$  cells/well) were cultured for the indicated times in 1 ml media containing no addition (●), 5  $\mu$ g/ml LPS (□), 500 U/ml IFN (○), or 500 U/ml IFN plus 1  $\mu$ g/ml LPS (Δ). The supernatants were analysed for nitrite/nitrate as described by Green *et al.* (1982). Values represent the mean of three wells. Insets show the course of experiments carried out over a longer time.

### Cycloheximide inhibition of nitrite/nitrate synthesis

The observed time lag suggested that protein synthesis was required before synthesis of nitrite/nitrate could take place. Therefore, we carried out experiments with the protein synthesis inhibitor cycloheximide. In C3H/He macrophages stimulated with  $\gamma$ -IFN and LPS over 48 h, cycloheximide inhibited nitrite/nitrate synthesis in a dose-dependent manner. Maximal inhibition was observed with 0.5  $\mu$ g/ml. Although cycloheximide was toxic at this dose, the decrease in viability does not explain the nearly 100% inhibition of synthesis. When 0.5  $\mu$ g/ml cycloheximide was added at different times after stimulation cells with LPS and  $\gamma$ -IFN, differential blocking was seen. When given in the 0-8-h period, cycloheximide completely blocked nitrite/nitrate synthesis. When macrophages were

stimulated for 8 h and then given cycloheximide, synthesis was not blocked and approximately the same amount was produced as in the first 8-h period; this was also true if cycloheximide treatment was delayed until 17-25 h. Although cycloheximide blocked macrophage nitrite/nitrate synthesis when given in the 0-8-h period, the macrophages were still capable of producing nitrite/nitrate if cycloheximide was removed at 8 h by washing. These results indicated that protein synthesis is required for synthesis of nitrite/nitrate by macrophages and that the relevant synthesis occurs within the first 8 h of activation. It is not yet possible, however, to distinguish the effects of cycloheximide on protein synthesis involved in macrophage activation in general and that required exclusively for nitrite/nitrate synthesis.

### **Role of the oxygen burst in macrophage synthesis of nitrite and nitrate**

Macrophages can be triggered to produce reactive oxygen species (ROI), such as superoxide, hydrogen peroxide and hydroxyl radical, when exposed to appropriate phagocytic, receptor or membrane stimuli. Furthermore, macrophages treated with agents such as LPS and IFN become primed to secrete increased amounts of ROI upon additional exposure to triggering signals, such as opsonized particles or phorbol myristate acetate (PMA; Johnston *et al.*, 1978). Since IFN- and LPS-activated macrophages also synthesize nitrite/nitrate, we were interested in determining if ROI were involved.

A membrane-associated NADPH oxidase is responsible for the oxygen burst in macrophages. Investigations on the role of this enzyme system in various macrophage functions have been aided by the generation of two cell lines, identical in all respects except that one is deficient and the other proficient in the ability to generate ROI. Further characterization has revealed that the mutation involves NADPH oxidase exclusively (Damiani *et al.*, 1980). Such cells could potentially be used to determine if ROI are involved in nitrite/nitrate synthesis.

The cells were obtained from Dr B. Bloom of Albert Einstein College of Medicine (NY, USA), and experiments were carried out to compare nitrite/nitrate synthesis by the superoxide-proficient J774.16 cell line with that of the superoxide-deficient J774 C3C cell line in response to LPS, IFN and IFN plus LPS (Table 1), and to measure coincident superoxide production by either cell line (using PMA). J774.16 and J774 C3C cells produced similar amounts of nitrite/nitrate when treated with IFN plus LPS. Nitrite represented approximately 60% of the total produced by either cell line. J774.16 cells produced superoxide when stimulated with PMA, whereas J774 C3C cells did not, when superoxide production was measured either as an initial rate in cell suspensions or over 60 min. The values for superoxide production by these cell lines agree with published data (Damiani *et al.*, 1980).

These results indicate that a functional membrane-associated superoxide generating system is not required for production of nitrite/nitrate by macrophages, suggesting that the oxygen burst is not involved. Scavengers of ROI, namely, superoxide dismutase, catalase and mannitol, had no effect on nitrite/nitrate synthesis in either cell line when added at time 0 or at 15 h after stimulation with LPS and IFN.

Although LPS and IFN can activate macrophages, they are not sufficient signals for ROI generation in and of themselves (Johnston *et al.*, 1978). We therefore tested if eliciting an oxygen burst (with PMA treatment) would increase nitrite/nitrate synthesis by macrophages activated previously by IFN plus LPS. To do this, J774.16 and J774 C3C macrophages were treated with IFN plus LPS for 15 h (a time sufficient for nitrite/nitrate

**Table 1. Comparison of nitrite/nitrate synthesis and superoxide production by J774.16 and J774 C3C macrophages<sup>a</sup>**

Stimulus	J774.16	J774 C3C
	Nitrite/nitrate synthesis (nmol)	
IFN + LPS	127 ± 14	118 ± 8
IFN	9 ± 4	12 ± 4
LPS	9 ± 4	23 ± 3
Control	0 ± 3	0 ± 1
PMA	Superoxide production (nmol/min per 10 <sup>6</sup> cells)	
	2.4 ± 0.1	0.0 ± 0.1
PMA	Superoxide production (nmol/60 min)	
	7.1 ± 0.2	0.4 ± 0.3

<sup>a</sup>Macrophages (1 × 10<sup>6</sup> per ml) were treated with IFN (500 U/ml), LPS (5 µg/ml) or IFN plus LPS (1 µg/ml) for 72 h. The initial rate of superoxide production was determined using suspensions of unstimulated cells (5 × 10<sup>5</sup> per ml) in a cuvette containing buffer, cytochrome c, and PMA (6 µg/ml). Superoxide produced in 60 min was determined using macrophage monolayers (1 × 10<sup>6</sup>) in 1 ml buffer containing cytochrome c ± PMA (µg/ml).

data indicate that the oxygen burst is not significantly involved in nitrite/nitrate synthesis, consistent with previously presented data.

**Table 2. Effect of triggering the oxygen burst on subsequent nitrite/nitrate production by J774.16 and J774 C3C macrophages activated by IFN plus LPS<sup>a</sup>**

Treatment	Nitrite/nitrate produced (nmol/10 <sup>6</sup> cells)		Superoxide produced (nmol/10 <sup>6</sup> cells)	
	J774.16	J774 C3C	J774.16	J774 C3C
IFN/LPS 15 h	74 ± 2	112 ± 5	-	-
IFN/LPS 15 h, then 3 h with:				
PMA	86 ± 5	125 ± 1	2.9 ± 0.1	0.0 ± 0.2
PMA plus superoxide dismutase	82 ± 9	125 ± 7	0.8 ± 0.1	-
Medium alone	85 ± 6	128 ± 7	0.0 ± 0.1	-

<sup>a</sup>Macrophages (1 × 10<sup>6</sup> per ml, 1 ml) were cultured for 15 h in media containing IFN (500 U/ml) and 1 µg/ml LPS. At this point, some of the supernatants were harvested, while other wells were cultured for an additional 3 h with the indicated treatments. Superoxide production by 15-h IFN/LPS-treated monolayers was measured using a cytochrome c assay (60 min). All values are the mean of three to four wells.

synthesis to occur, after which a portion were treated with PMA ± superoxide dismutase for an additional 3 h (Table 2). Both cell lines had produced a significant amount of nitrite/nitrate by 15 h. Further incubation for 3 h resulted in approximately equivalent amounts of additional synthesis, irrespective of the presence of PMA. Table 2 also shows that addition of PMA caused superoxide production by J774.16 macrophages that had been treated for 15 h with IFN plus LPS, and that superoxide dismutase scavenged superoxide when present. Similar experiments done with thioglycolate-elicited mouse macrophages (C3H/He) yielded identical results. J774.16, J774 C3C and C3H/He macrophages treated with PMA alone for 15 or 24 h did not produce nitrite/nitrate, although an oxygen burst occurred in all cases. Taken together, these

#### Acknowledgements

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## N-NITROSAMINE FORMATION BY MACROPHAGES

M. Miwa, D.J. Stuehr, M.A. Marletta, J.S. Wishnok & S.R. Tannenbaum<sup>1</sup>

*Massachusetts Institute of Technology, Department of Applied Biological  
Sciences, Cambridge, MA 02139, USA*

Nitrate biosynthesis is a known mammalian process, and macrophages from mice treated with *Escherichia coli* lipopolysaccharide (LPS) have been shown to be capable of nitrate synthesis. Cell culture studies showed that macrophages produce nitrite as well as nitrate. We report here *N*-nitrosamine formation by stimulated macrophages. Experiments were carried out with the macrophage cell lines, J774.1, WEHI-3 and RAW 264. Macrophages were cultured in Dulbecco's modified Eagle's medium (pH 7.5) supplemented with calf serum (10%). The concentration of nitrate in the supernatant was measured. *N*-Nitrosamines were extracted with dichloromethane and the extracts were analysed by gas chromatography-thermal energy analysis. When J774.1 ( $1.5 \times 10^6$  cells/ml) were incubated with LPS (10  $\mu$ g/ml) and morpholine (15 mM) for 72 h at 37°C, *N*-nitrosomorpholine (NMOR) was produced (0.8  $\mu$ M). The amount of nitrite produced was 50  $\mu$ M. RAW 264 and WEHI-3 also produced NMOR; LPS was required for nitrite and NMOR formation.  $\gamma$ -Interferon (IFN) promoted both NMOR (2.5  $\mu$ M) and nitrite (70  $\mu$ M) formation. Nitrite (150  $\mu$ M) incubated with morpholine and the medium did not form NMOR. Kinetics of LPS-induced nitrite and NMOR formation in J774.1 showed that the rate of NMOR formation was highest in the middle incubation period (24-36 h), although the nitrite concentration was highest in the latter incubation period (48-60 h). Our results showed that macrophages may be capable of nitrosamine formation under physiological conditions that do not normally permit this reaction.

In a recent series of papers, we elaborated the mechanisms of nitrate biosynthesis in mammalian animals and cells (Green *et al.*, 1981; Wagner *et al.*, 1983b; Stuehr & Marletta, 1985). As a result of the experiments in cell culture, it became apparent that stimulated macrophages produced nitrite and nitrate in a constant ratio (approximately 3:2 M/M). In whole animal experiments, nitrite could not be observed, because of immediate oxidation of nitrite to nitrate by oxyhaemoglobin. However, it is reasonable to assume that 60% of the nitrate formed by endogenous processes *in vivo* was originally formed as nitrite. This could amount to 300  $\mu$ mol per day of nitrite in the average infection-free person (Green *et al.*, 1981) and up to 2 or more mmol per day in the case of viral or bacterial infection (Wagner & Tannenbaum, 1982).

By any standard, this is a large quantity of nitrite. If this nitrite participated in nitrosation reactions prior to conversion to nitrate, even a small yield of *N*-nitroso compound would represent a significant addition to the existing body burden. In this connection, it is not unreasonable to speculate that the basal, nongastric synthesis of

<sup>1</sup>To whom correspondence should be addressed

*N*-nitrosoproline in humans is related to nitrite synthesis by macrophages. We have estimated this basal level to be of the order of 20-30 nmol/day (Wagner *et al.*, 1983b), which would be approximately  $10^{-4}$  of the amount of nitrite. In this paper we present the first evidence of nitrosamine synthesis by macrophages *in vitro*, in support of the speculations discussed above.

### Materials and methods

**Cells:** Experiments have been conducted with a variety of immortalized macrophage cell lines (reported here are J774, WEHI-3 and RAW264) as well as peritoneal macrophages from C3H/He mice elicited by thioglycolate injection. Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% calf serum (HyClone, Logan, UT, USA) was used to maintain the cell lines. For each experiment, minimum essential medium (without phenol red, Flow) supplemented with sodium bicarbonate (2.0 g/l), glucose (3.5 g/l), sodium pyruvate (0.11 g/l), HEPES (10 mM), penicillin (5 international units/ml)/streptomycin (5  $\mu$ g/ml) and 10% calf serum was used in order to avoid interference in nitrite analysis by phenol red.

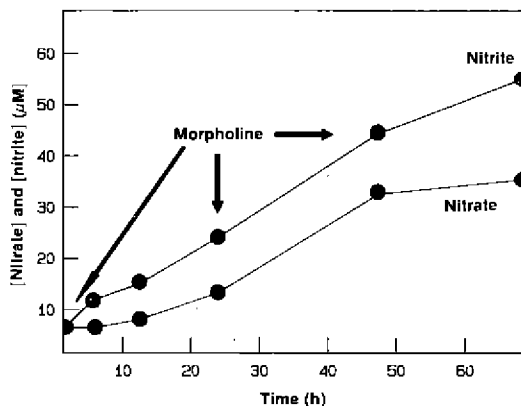
The macrophages were plated ( $1 \times 10^6$  cells/ml) and incubated for 1 h at 37°C in 5% carbon dioxide/95% air to allow for macrophage adherence. The supernatant was then removed, and appropriate experimental medium was added. The variables in the experiments included LPS, IFN and the appropriate amine, which were added in various combinations according to the experiment.

**Analytical method:** Nitrite in cell-culture supernatant samples was measured by reaction with the Griess reagent (1% sulfanilamide in 5% phosphoric acid/0.1% naphthylethylenediamine dihydrochloride) to form a chromophore absorbing at 540 nm.

For analysis of NMOR, 200  $\mu$ l 5 N sodium hydroxide were added to 200  $\mu$ l cell culture supernatant to stop the reaction. Then, 200  $\mu$ l dichloromethane were added, and an aliquot of dichloromethane extract was analysed by gas chromatography interfaced with a Thermal Energy Analyzer, model 502 (Thermal Electron Corp., Waltham, MA, USA). For the other nitrosamines, the reaction was stopped by adding 15 ml of 5 N sodium hydroxide to the 15 ml of medium, and then a suitable amount of internal standard (*N*-nitrosodipropylamine) was added to the mixture. The nitrosamines were extracted with dichloromethane (30 ml  $\times$  2), and dichloromethane extracts were concentrated in a Kuderna-Danish evaporator at 52°C to 10 ml and then under nitrogen to 300  $\mu$ l. An aliquot was analysed in the same way as NMOR.

### Results and discussion

It became rapidly apparent from our initial experiments that stimulated macrophages were capable of nitrosating a variety of acceptor amines under physiological conditions (pH well-buffered at 7.4, a high concentration of serum proteins). Figure 1 gives a typical set of results for nitrate/nitrite production and, additionally, illustrates the experiment described below and in Table 1. Cells were stimulated with LPS at 0 time; morpholine was added at 0 or 24 and 48 h later. Aliquots were taken for analysis of NMOR 12 h following addition of morpholine and therefore represent the amount of nitrosamine synthesis in that 12-h time period. As the figure shows, nitrite and nitrate were synthesized continuously over the 72-h period. The maximum rate of NMOR synthesis, however, corresponds to the maximum rate of nitrite formation and not to the maximum concentration of nitrite (Table 1). These results suggest a model in which the nitrosamine forms in parallel with the formation of nitrate and nitrite.

**Fig. 1. LPS-induced synthesis of nitrate and nitrite****Table 1. Nitrosation of morpholine at various times during nitrite/nitrate production**

Incubation period (h)	NMOR (nM)	Nitrite (μM)
0 - 12	ND	2.4
24 - 36	509	32.4
48 - 60	121	76.7

ND, not detected

This model is in turn supported by experiments in which nitrite added to macrophage cultures in the presence of morpholine fail to produce NMOR. In Table 2, results are presented for a series of experiments designed to test the possibility that nitrosamine formation is an artefact in this system. Although small amounts of NMOR form in the presence of high concentrations of nitrite, even at physiological pH, only the combination of cells plus LPS plus morpholine leads to a significant increase in NMOR.

In Tables 1 and 2, the yield of NMOR can be seen to be approximately  $10^{-2}$  to  $10^{-3}$  of the nitrite yield over more than one order of magnitude of morpholine concentration. There is no influence of morpholine concentration on nitrite formation (Fig. 2). This again supports a model in which NMOR forms in parallel to nitrite and nitrate.

**Table 2. NMOR formation by J774 macrophages**

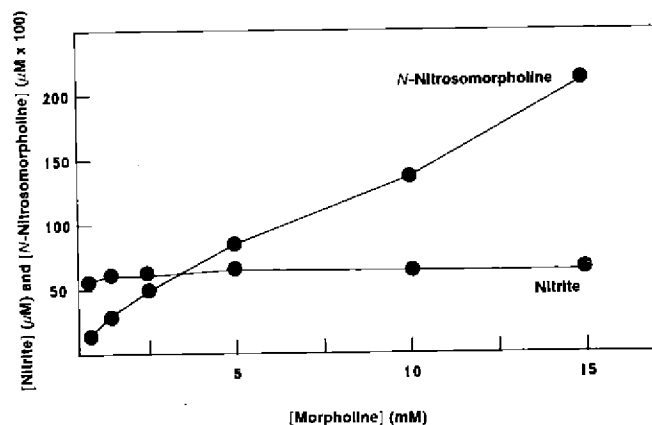
Cells ( $1 \times 10^6$ /ml)	LPS (10 μg/ml)	Morpholine (15 mM)	NMOR (nM)	Nitrite (μM)
-	+	+	ND	ND
+	-	+	ND	15.2
+	+	+	594	45.0

Cells	Nitrite (150 mM)	Morpholine (15 mM)	NMOR (nM)
-	+	+	78

ND, not detected

**Fig. 2. Effect of morpholine concentration on formation of nitrite and NMOR**



IFN enhanced the rate of formation of nitrate/nitrite and NMOR to about the same degree as LPS. The effect of LPS and IFN in combination was 3-4 times greater than that of either substance alone.

Extension of these experiments to other amines is shown in Table 3. The experiments shown for diethylamine, dibutylamine and methylbenzylamine are complicated by the fact that these amines proved toxic to the cells at the higher concentration. Nevertheless, each of the amines showed significant nitrosamine formation after 72 h of stimulation

with LPS plus IFN. The results suggest that the free base in the system is probably the species being nitrosated (NMOR > *N*-nitrosomethylbenzylamine > *N*-nitrosodibutylamine), but the lipophilicity of the amine may also be important (*N*-nitrosodibutylamine > *N*-nitrosodiethylamine).

**Table 3. *N*-Nitrosation of amines by RAW 264 (72-h incubation)**

Amine	(mM)	Ingredients			[Nitrite] (μM)	[Nitrosamine] (nM)
		Cells	LPS	IFN		
Diethylamine	10	-	+	+	2.9	5
	10	+	-	-	4.8	9
	10	+	+	+	36.6	9
	5	+	+	+	57.1	9
Dibutylamine	10	-	+	+	1.4	15
	10	+	-	-	6.9	21
	10	+	+	+	24.4	18
	5	+	+	+	76.5	38
Methylbenzylamine	10	-	+	+	6.0	12
	10	+	-	-	5.7	6
	10	+	+	+	28.9	180
	5	+	+	+	60.0	267
Morpholine	5	+	+	+	59.3	1680

**Conclusions**

- (1) LPS-stimulated macrophages can synthesize *N*-nitroso compounds.
- (2) LPS plus IFN is a more potent stimulus than LPS alone.
- (3) The yield of nitrosamine is proportional to the rate of nitrite formation and the concentration of free amine.
- (4) The possibility exists that *N*-nitroso compounds may be formed endogenously *via* activated macrophages.

**Acknowledgements**

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# PEPTIDE NITROSION IN DILUTE ACID

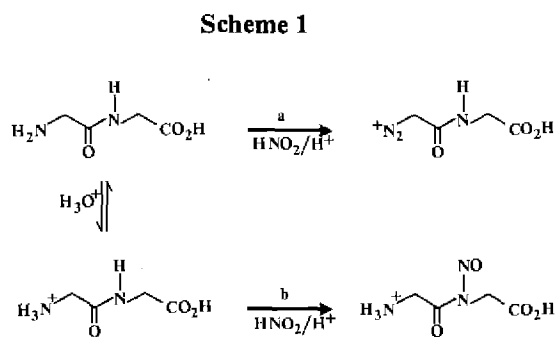
B.C. Challis<sup>1,3</sup>, B.R. Glover<sup>1</sup> & J.R.A. Pollock<sup>2</sup>

<sup>1</sup>Chemistry Department, Imperial College, London SW7 2AZ; and

<sup>2</sup>Pollock and Pool Ltd, Ladbroke Close, Woodley, Reading RG5 4DX, UK

Rates and products are reported for the nitrosation of simple dipeptides (glycylglycine, its ethyl ester and *N*-acetylglycylglycine) in dilute acid at 37°C. The results suggest that conversion to a diazo derivative (which rapidly decomposes) is the most likely outcome of the gastric nitrosation of small proteins and peptides.

Proteins and peptides are common dietary constituents and therefore likely substrates for endogenous nitrosation. Reaction may occur at either the terminal or peptide *N*-atoms (Scheme 1) as well as at other sites in substrates containing cysteine, tryptophan, tyrosine, asparagine, glutamine, lysine and arginine residues.



Nitrosations at terminal *N*-atoms (path a, Scheme 1) to give either a diazopeptide (Curtius & Thompson, 1906; Kurosky & Hofmann, 1972) or an *N*-nitroso derivative (Kubacka *et al.*, 1984; Kubacka & Scanlan, 1984) are well documented. With excess nitrite, diazopeptides rearrange and form *N*-nitrosoiminodialkanoic acids (Pollock, 1985). *N*-Nitrosopeptides (path b, Scheme 1) were synthesized recently and shown to form in aqueous acid (Challis *et al.*, 1984).

There is little information, however, on the relative importance of each pathway in Scheme 1 under gastric conditions. To remedy this deficiency, we have investigated the nitrosation of some simple dipeptides in dilute aqueous acid at 37°C.

## Kinetic studies

The kinetics of peptide nitrosation in dilute acid were investigated using glycylglycine, a simple, readily available substrate. The reactions were followed by measuring the uptake of nitrite colorimetrically by Shinn's procedure (Kershaw & Chamberlin, 1942). Allowance

<sup>3</sup> To whom correspondence should be addressed

was made for the concurrent, thermal decomposition of nitrite. Glycylglycine ethyl ester was also examined in connection with product quantification by gas-liquid chromatography-thermal energy analysis.

In dilute HCl at 37°C, the nitrosation of glycylglycine followed kinetics (equation 1) typical of an amine.

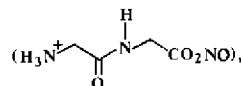
$$\text{Rate} = k_3[\text{Glygly}][\text{Nitrite}][\text{Cl}^-] \quad (1)$$

Variation of  $\log t_{1/2}$  with  $\log [\text{Nitrite}]$  in Figure 1 shows the first-order nitrite dependence applied for a wide range ( $10^{-2}$  -  $10^{-5}$  M) of concentrations. Equation 1 is that expected for reaction of the neutral peptide with NOCl.

In dilute HClO<sub>4</sub> at 37°C, however, the kinetics (equation 2) were more complicated, in two ways.

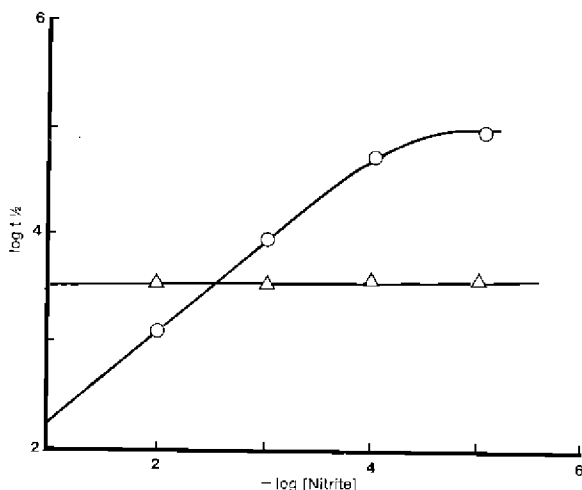
$$\text{Rate} = [\text{Glygly}][\text{Nitrite}] \{k'_2 + k'_3[\text{Nitrite}] + k''_3[\text{Glygly}]\} \quad (2)$$

Firstly, the nitrite dependence decreased from second- to first-order (Fig. 1) over the range  $10^{-2}$  -  $10^{-5}$  M nitrite. Secondly, the glycylglycine dependence, while being first-order at  $10^{-2}$  M nitrite, increased from first- to second-order with increasing [Glygly] at  $10^{-4}$  -  $10^{-5}$  M nitrite: this difference is seen clearly in Figure 2. The  $k'_2$  and  $k'_3$  terms of equation 2 were expected, corresponding to reaction of the neutral peptide with N<sub>2</sub>O<sub>3</sub> and NO<sup>+</sup>, respectively. The  $k''_3$  term of equation 2 was unexpected, but probably related to an intermolecular nitrosation of the neutral peptide by the nitrite ester of glycylglycine



because a similar term was not apparent for glycylglycine ethyl ester (Fig. 2).

**Fig. 1.** Variation of  $\log t_{1/2}$  with  $-\log[\text{Nitrite}]$  for the nitrosation of 0.1 M glycylglycine at 37°C in HClO<sub>4</sub> (pH 3.2) (○) and HCl (pH 1.3) (Δ)

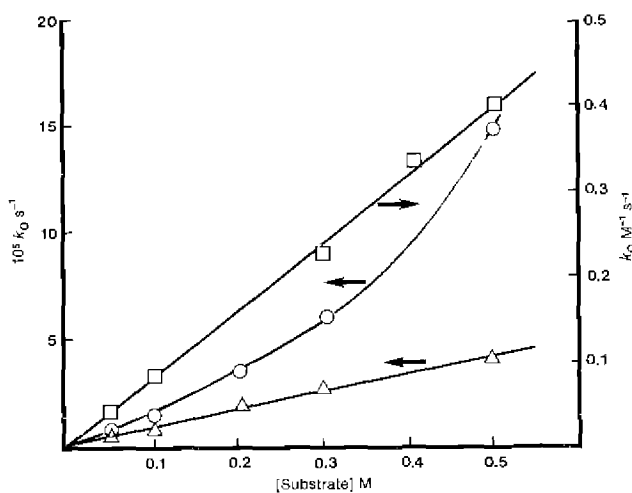


The results suggest that under normal gastric conditions (37°C, pH 1-4, [Nitrite]  $\leq 20 \times 10^{-6}$  M, [Cl<sup>-</sup>] =  $10^{-1}$  -  $10^{-4}$  M), the nitrosation of glycylglycine follows equation 3 with only a first-order nitrite dependence. Further, the rate of nitrosation ( $k_2$  value) is dependent on [Cl<sup>-</sup>],

$$\text{Rate} = k_2[\text{Glygly}][\text{Nitrite}], \quad (3)$$

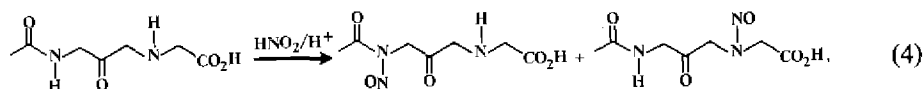
but virtually independent of acidity below pH 3 (Fig. 3). These observations are at variance with the second-order nitrite- and pH-dependent kinetics often *assumed* for the nitrosation of amine compounds under gastric conditions.

Fig. 2. Effect of [Substrate] on rates of nitrosation at pH 3.2 and 37°C: ○, glycylglycine plus  $10^{-4}$  M nitrite; △, glycylglycine ethyl ester plus  $10^{-4}$  M nitrite; □, glycylglycine plus  $10^{-2}$  M nitrite



### *N*-Acetylglycylglycine

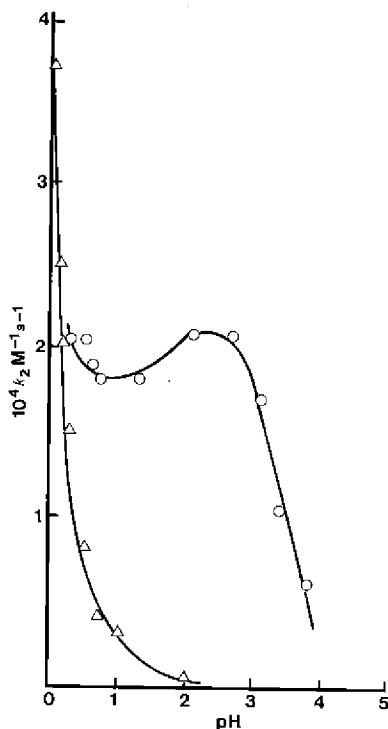
*N*-Acetylglycylglycine was tested to assess the relative reactivity of peptide and terminal *N* atoms. This compound has two *N* atoms in a similar chemical environment to the single peptide *N* atom of glycylglycine. Thus, its rate of nitrosation should be about twice that of the peptide *N* atom of glycylglycine. Previously, *N*-acetylglycylglycine was shown to produce two *N*-nitroso derivatives (equation 4),



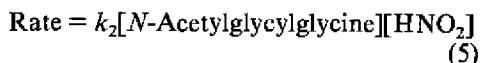
both of which decomposed in dilute acid to deamination products (Challis *et al.*, 1984). The nitrosation was therefore followed (as for glycylglycine) by the uptake of nitrite using Shinn's procedure (Kershaw & Chamberlin, 1942), making allowance for concurrent, thermal decomposition of nitrite.



**Fig. 3. Acidity dependencies for the nitrosation of 0.1 M glycylglycine (○) and 0.1 M *N*-acetylglycylglycine (△) by  $10^{-4}$  M nitrite at 37°C**



The nitrosation kinetics for *N*-acetylglycylglycine were simpler than those for glycylglycine. Irrespective of the conditions, the reaction showed just first-order dependencies on both *N*-acetylglycylglycine and  $\text{HNO}_2$  (equation 5) and was strongly acid catalysed (Fig. 3). These are the expected dependencies for the nitrosation of an *N*-acyl compound and probably reflect rate-limiting  $\text{H}^+$ -loss from the protonated *N*-nitroso intermediates.



Examination of Figure 3 shows that in the normal gastric range of pH 1-4 with  $10^{-4}$  M nitrite, diazotization of glycylglycine is at least 20 times faster than nitrosation of its peptide *N* atom. Further, diazotization was more strongly catalysed by  $\text{Cl}^-$  and  $\text{SCN}^-$  than peptide nitrosation. In 1 M  $\text{HClO}_4$  with  $10^{-4}$  M nitrite at 37°C,  $10^{-3}$  M  $\text{SCN}^-$  and 1.0 M  $\text{Cl}^-$  increased the rate of diazotization of glycylglycine by factors of 170 and 50, respectively, but the nitrosation of *N*-acetylglycylglycine by factors of about two only. Thus, for small proteins and peptides, conversion to a diazo derivative seems to be the most likely outcome of gastric nitrosation.

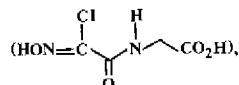
### Product analysis

Both diazo peptides ( $\lambda_{\text{max}}$ , about 250 nm;  $\log \epsilon$ , about 4.3) and *N*-nitrosopeptides ( $\lambda_{\text{max}}$ , about 240 nm;  $\log \epsilon$ , about 3.8) have well-defined ultra-violet absorbances. Spectrophotometric examination of reaction solutions showed little evidence of either product, even though nitrite had been consumed. This was not unexpected, however, because both compounds decompose in aqueous acid. The reaction solutions were therefore examined for decomposition products.

The kinetic studies implied that the major decomposition products should derive from a diazo peptide. This was confirmed by comparing the products from the nitrosation of glycylglycine ethyl ester with those from the decomposition of authentic *N*-diazoacetylglycine ethyl ester under identical conditions. The ethyl ester was used to improve product volatility and it reacted at virtually the same rate as glycylglycine itself. Conditions were chosen to give much shorter reaction times ( $t_{1/2}$ , about 10 min) than the thermal decomposition of nitrite ( $t_{1/2}$ , about 500 min). The reaction solutions were assayed after 60 min (i.e., six half-lives) by capillary gas-liquid chromatography (Carlo Erba 4185) coupled to a Thermal Energy Analyzer (Thermo-Electron 610) (GLC-TEA) against authentic compounds. The data were corrected for sub-100% recoveries from these authentic compounds.

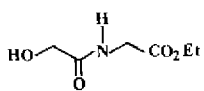
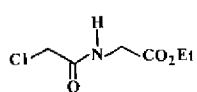
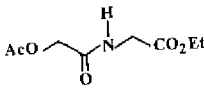
Table 1 shows, with excess glycylglycine ethyl ester, the major products derived from interaction of the diazopeptide with  $H_2O$ ,  $Cl^-$  or  $AcO^-$ . No evidence was found for the formation of *N*-nitrosoiminodiacetic acid esters. *N*-Nitrosoiminodialkanoic acids were found, however, for the nitrosation of 0.1 M L-alanyl-L-alanine and 0.1 M L-phenylalanylglycine in 0.1 M HCl at 25°C (Table 2). These products were quantified by GLC-TEA, following the procedure of Pollock (1985). For gastric levels of nitrite, the yields of *N*-nitrosoiminodialkanoic acids were below 1%.

Three other minor products were apparent in reactions of glycylglycine with excess nitrite. One (10% yield) was identified as the oxime but the



other two (1-2% yields) have yet to be characterized.

**Table 1. Products (% yield)<sup>a</sup> from the nitrosation of 0.1 M glycylglycine ethyl ester and the decomposition of *N*-diazooacetyl glycine ethyl ester at 37°C**

[Nitrite] M	1 M HCl				1 M AcOH buffer
	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	10 <sup>-3</sup>
	33(33)	34(36)	42(45)	67(63)	68(68)
	51(59)	55(56)	46(47)	39(49)	
	-	-	-	-	31(31)
Totals	84(92)	89(92)	88(92)	106(112)	99(99)

<sup>a</sup>Based on nitrite. Those in parentheses are for the decomposition of 10<sup>-2</sup> - 10<sup>-5</sup> M *N*-diazooacetyl glycine ethyl ester.

**Table 2. Yields of *N*-nitrosoiminodialkanoic acids from reaction of 0.1 M L-alanyl-L-alanine or 0.1 M L-phenylalanylglycine with nitrite in 0.1 M HCl at 25°C**

10 <sup>4</sup> [Nitrite]	% Yield <sup>a</sup>	
	[HO <sub>2</sub> CCH(Me)] <sub>2</sub> NNO	HO <sub>2</sub> CCH(CH <sub>2</sub> Ph)N(NO)CH <sub>2</sub> CO <sub>2</sub> H
2000	9.5	4.9
1000	5.2	6.6
100	0.37	1.7
10	0.19	0.26
1	0.09	0.59

<sup>a</sup>Based on nitrite

### Conclusions

In dilute acid at 37°C, simple dipeptides react readily with nitrite ( $t_{1/2}$ , 1-20 h, depending on concentrations and conditions). Under simulated normal gastric conditions (pH 1-4,  $[Cl^-] = 10^{-1} - 10^{-4}$  M,  $[Nitrite] \leq 20 \times 10^{-6}$  M, 37°C), the reactions show a first-order dependence on nitrite and are virtually independent of acidity below pH 3. These dependencies are at variance with the second-order nitrite and pH-dependent kinetics usually assumed for the endogenous nitrosation of amine compounds. Both the kinetic and product studies show that formation of a diazo rather than an *N*-nitroso derivative is the major pathway for the nitrosation of dipeptides in dilute acid. This suggests that conversion to a diazo derivative is the most likely outcome of the gastric nitrosation of small proteins and peptides. Since these diazo derivatives are unstable, readily losing nitrogen in dilute acid at 37°C, small proteins and peptides should inhibit the formation of *N*-nitroso compounds in the stomach. Some diazo derivatives of dipeptides rearrange and react further to *N*-nitrosoiminodialkanoic acids. With normal gastric levels of nitrite ( $< 20 \times 10^{-6}$  M), the yields of *N*-nitrosoiminodialkanoic acids are below 1%.

### Acknowledgements

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# NITROSATION OF DRUGS UNDER IN-VIVO CONDITIONS

C.L. Walters<sup>1,4</sup>, P.N. Gillatt<sup>2</sup>, R.C. Palmer<sup>2</sup>, P.L.R. Smith<sup>2</sup>  
& P.I. Reed<sup>3</sup>

<sup>1</sup>Department of Biochemistry, University of Surrey, Guildford, Surrey; <sup>2</sup>Leatherhead Food Research Association, Leatherhead, KT22 7RY; and <sup>3</sup>Gastrointestinal Unit, Wexham Park Hospital, Slough, Berkshire, UK

Application of the WHO Nitrosation Assay Procedure (NAP test) to a range of potentially nitrosatable drugs has given rise to considerable variations in the formation of volatile *N*-nitrosamines and *N*-nitroso compounds as a group. No nitrosation whatsoever was observed with 40 mM nitrite in some instances. In simulating more closely the conditions likely to be encountered in the human stomach, however, the order of susceptibility of the drugs to *N*-nitrosation has proved to be very different. At a constant nitrite concentration of 25  $\mu$ M, which is considered to represent the upper limit of those likely to be encountered in the acidic human stomach, the drugs giving rise to the greatest yields of products reacting as *N*-nitroso compounds from a maximum adult dose were the penicillins, G, V, cloxacillin and ampicillin.

The NAP test has been proposed by Coulston and Dunne (1980) as a means of ranking drugs on the basis of their susceptibilities to reaction with nitrite at pH 3 to form potentially carcinogenic *N*-nitroso compounds. Its conditions, however, are far removed from those appertaining to human gastric juice, and, in consequence, the extents of nitrosation using a range of drugs have been compared with those obtained under conditions considered to be more appropriate to the fasting stomach.

## Sources of drugs

Chlorpheniramine and chlordiazepoxide were kindly provided by Dr W. Lijinsky of the Frederick Cancer Research Laboratory, PO Box B, Frederick, MD 21701, USA. Mepyramine maleate and trimeprazine tartrate were donated by May & Baker Ltd, of Dagenham, Essex, UK, timolol maleate by Merck, Sharp & Dohme, of Hoddesdon, Hertfordshire, UK and naratriptyline hydrochloride by E. R. Squibb & Sons Ltd, of Moston, Merseyside, UK. All other drugs were purchased as of clinical grade from pharmaceutical suppliers and mainly from Sygma London Chemical Company of Parkstone, Dorset, UK.

## Choice of conditions to simulate those relevant to the fasting human stomach

Drugs were dissolved or suspended in simulated gastric juice (Long *et al.*, 1961), buffered to pH 2.0, to a level of one normal maximum adult dose per 50 ml. Incubations at 37°C were carried out over 1 h and 3 h with continuous shaking and protection from light. In the absence of a drug, an initial concentration of 25  $\mu$ M nitrite was found to decay

<sup>4</sup> To whom correspondence should be addressed

exponentially, and nitrite was added dropwise to counteract this decomposition. In the presence of a drug, it was necessary to determine the nitrite levels by the rapid chemiluminescence method of Walters *et al.* (1986) and to restore them at 15-min intervals to 25  $\mu\text{M}$ , which is considered to represent the upper limit in the normal fasting stomach, to simulate its replenishment from the saliva *in vivo*. Volatile *N*-nitrosamines were determined by gas chromatography with a Thermal Energy Analyzer (TEA) as detector, and total *N*-nitroso compounds by the group-selective procedure of Walters *et al.* (1978).

#### **Volatile *N*-nitrosamines derived from drugs during the NAP test**

Of the ten drugs capable of producing volatile *N*-nitrosamines on reaction with nitrite, aminopyrine and minocycline were by far the most readily subject to nitrosative cleavage. Determinations of *N*-nitroso compounds as a group showed that *N*-nitrosodimethylamine (NDMA) was the only product of reaction under NAP conditions, although in each case other types of compounds were formed which decomposed to nitric oxide on heating alone; such behaviour is exhibited by pseudonitrosites formed across double bonds. Much lower yields of NDMA were obtained from chlortetracycline and oxytetracycline, while those of volatile *N*-nitrosamines from the other relevant drugs were all less than 1%. These included NDMA from promethazine, chlorpromazine and imipramine, *N*-nitrosodiethylamine from disulfiram and *N*-nitrosomorpholine from timolol. No volatile *N*-nitrosamine was detected in the product from procaine, and that from chlorpheniramine was not studied under NAP conditions.

#### **Comparison of yields of *N*-nitroso compounds as a group from drugs under simulated gastric conditions and those of the NAP test**

Table 1 shows the conversions of a range of drugs into their *N*-nitroso derivatives when a maximum recommended dose (Reynolds, 1982) of each was subjected to simulated gastric conditions for 3 h; the values for an incubation period of 1 h were approximately three times less. The table also brings together for comparison results reported independently for both simulated gastric and NAP conditions (Gillatt *et al.*, 1984, 1985). The highest yield of putative *N*-nitroso compounds, on the basis of dosage prescribed, was obtained from penicillin G: no less than 45% of the nitrite supplied to maintain its concentration at 25  $\mu\text{M}$  was located in the form of compounds yielding nitric oxide on denitrosation, in the manner of *N*-nitroso compounds.

Although the sodium salt of penicillin G is generally administered by injection and would therefore not enter the stomach directly, its benzathine salt, which is nitrosated even more readily under simulated gastric conditions, is available for oral administration; the recommended adult dose in mild infection is 450 mg every 6-8 h, with half this amount for children (Reynolds, 1982). Penicillin V is more resistant than penicillin G to acidic gastric secretions, and in consequence its oral administration is most common.

In almost all instances, drugs that formed products with the properties of *N*-nitroso compounds under NAP conditions continued to do so under the simulated gastric conditions adopted, albeit to much smaller extents. In general, the tendency for more facile nitrosation of secondary than that of tertiary amines (with aminopyrine and minocycline as exceptions) was less apparent using a realistic dose at the lower nitrite level; under these conditions, for instance, the tertiary amine, cyclizine, formed more putative *N*-nitroso compounds than the related secondary amine, piperazine, on a dose weight basis.

Table 1. *N*-Nitrosation of drugs under simulated gastric and NAP conditions

Order	Drug	Type of compound <sup>a</sup>	Dose (mg) <sup>b</sup>	Simulated gastric conditions		NAP conditions	Ratio <sup>d</sup> × 10 <sup>-3</sup>
				Nitroso compound (nmol)	Conversion of drug (%)	Conversion of drug (%)	
1	Penicillin G (Na salt)	C,D	250	3900	0.55	17	32
2	Penicillin V (K salt)	C,D	250	3300	0.50	6.8	7.4
3	Cloxacillin	C	250	1200	0.20	-	-
4	Ampicillin	C	250	420	0.06	-	-
5	Aminopyrine	B	600	340	0.010	47	0.21
6	Ethambutol	A	400	100	0.005	140	0.036
7	Cyclizine	B	50	60	0.030	0.19	1.6
8	Imipramine	B	75	55	0.020	0.24	83
9	Chlortetracycline	B	250	35	0.007	3.7	1.9
10	Piperazine	A	1000	35	0.0003	115	0.0026
11	Oxytetracycline	B	250	25	0.005	2.7	1.9
12	Epinephrine	A	60	25	0.008	10	0.80
13	Timolol	A,B	30	25	0.035	8.8	4.0
14	Minocycline	B	50	25	0.021	34	0.62
15	Propranolol	A	30	18	0.015	29	0.52
16	Nortriptyline	A	25	17	0.012	9.8	1.2
17	Chlorpromazine	B	100	16	0.005	0.23	21
18	Chlordiazepoxide	A	30	11	0.011	0.28	39
19	Promethazine	B	25	9.7	0.011	2.0	5.5
20	Chlorpheniramine	B	5	1.0	0.010	-	-
21	Pindolol	A	30	0	0	14	-
22	Nialamide	A,C,E	150	0	0	0.41	-
23	Phenytoin	C	100	0	0	0.12	-
24	Procaine	B	2000	0	0	0.010	-
25	Chlorthalidone	A	100	0	0	0	-

<sup>a</sup>A, secondary amine; B, tertiary amine; C, secondary amide; D, tertiary amide; E, hydrazide

<sup>b</sup>Normal maximal adult dose

<sup>c</sup>Based on one nitrosatable group per molecule

<sup>d</sup>Ratio of conversion under simulated gastric conditions to those under NAP conditions

It is apparent from the 30 000-fold variation in the ratio of the percentage conversion to putative *N*-nitroso compounds under simulated gastric conditions to those related to the NAP test that the latter dose does not represent a valid criterion as a measure of the nitrosatability of a drug when a realistic dose is employed and the nitrite level is comparable with the range encountered *in vivo*. Under such conditions, therefore, the nitrosation of aminopyrine, which is used in some countries at high dosage, is limited by the availability of nitrite, a situation which does not apply in the NAP test.

The penicillins may be administered to humans, including children, for long periods, and thus their capacity to form putative *N*-nitroso compounds could well be of concern, particularly if the products of reaction with nitrite are mutagenic. In preliminary studies undertaken by H. Bartsch and C. Malaveille at IARC, Lyon, France, for instance, the products of the reactions of penicillin G with nitrite under NAP test conditions have shown mutagenicity towards *Salmonella typhimurium* TA100 without metabolic activation.

#### **Acknowledgements**

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# IN-VITRO AND IN-VIVO FORMATION OF N-NITROSOMETHYLCYCLOHEXYLAMINE FROM BROMHEXIN AND SODIUM NITRITE, AND DNA METHYLATION IN RATS

K.H. Schlemmer & G. Eisenbrand

*Department of Food Chemistry and Environmental Toxicology,  
University of Kaiserslautern, 6750 Kaiserslautern, Federal Republic of Germany*

After oral administration of a commercial bromhexin (*N*-methyl-*N*-cyclohexyl(2-amino-3,5-dibrombenzyl)ammonium chloride) solution (1-90 mg/kg) together with sodium nitrite (1-90 mg/kg) to female Wistar rats, ring-hydroxylated metabolites of *N*-nitrosomethylcyclohexylamine (NMCA) were excreted in urine as glucuronide/sulfate conjugates. When [<sup>14</sup>C-methyl]-bromhexin (30 mg/kg) was given intragastrically together with sodium nitrite (30 mg/kg), alkylated DNA adducts were detected in liver and oesophagus. Gastric juice of 75 healthy human volunteers (fasted, then ingesting up to 200 mg nitrate) was incubated *in vitro* with bromhexin (16 mg/100 ml). In only one sample, 50 ng NMCA/100 ml were formed.

The antitussive and secretolytic drug bromhexin reacts under the conditions of the WHO-Nitrosation Assay Procedure (NAP) (Coulston & Dunne, 1980) with nitrite in aqueous solution to yield NMCA in high yields. NMCA is a potent oesophageal carcinogen in rats (Druckrey *et al.*, 1967).

## Materials and methods

*Formation of NMCA in vitro:* Bromhexin hydrochloride (1.0 mmol) was dissolved in acetic acid (50 ml) overnight. After addition of double-distilled water (20 ml), the pH was adjusted to 3.2 with 4 N sodium hydroxide. Sodium nitrite (4 mmol) was added and the volume made up with water to 100 ml; the mixture was incubated at 37°C. Aliquots (1 ml) were removed after 1 and 4 h, and nitrite was destroyed by addition of 2 M sulfamic acid (1 ml). Alternatively, further nitrosation was blocked by addition of 2 N sodium hydroxide. The solution was applied to an Extrelut<sup>®</sup>-column (5 g), and, after 20 min of equilibration, the column was extracted with pentane (30 ml). Eluates were concentrated to 10 ml under a stream of nitrogen.

In another *in-vitro* study, bromhexin hydrochloride (9.7 μmol) was incubated at 37°C for 1 and 4 h in a buffer solution (citrate/hydrochloric acid, pH 3) with various concentrations of sodium nitrite (2.2-22 μmol, corresponding to 1-10 ppm nitrite). Aliquots of 10 or 20 ml were made alkaline with 2 N sodium hydroxide (0.5 ml), saturated with ammonium sulfate and placed onto columns of about 10 g Extrelut<sup>®</sup>. After equilibration for 20 min, the columns were eluted with hexane (50 ml), and the eluates were reduced to a final volume by a stream of nitrogen.



In a further study, buffer solutions and gastric juice in the range of pH 2-7 and nitrite levels in the range of 0.4-50  $\mu\text{g}/\text{ml}$  sodium nitrite were incubated with bromhexin (160  $\mu\text{g}/\text{ml}$ ) at 37°C for 1 h and worked up as described above.

NMCA was identified by gas chromatography (GC)-mass spectrometry: GC column, OV 101 3%, 1.2 m, 140°C. The determination was performed by GC with a thermal energy analyser (TEA) as detector (GC-TEA); column: OV 101 3%, 1.2 m, 140°C; alternatively: OV 351 6%, 1.2 m, 160°C.

*Detection of ring-hydroxylated NMCA metabolites (OH-NMCA) in rat urine after administration of bromhexin and sodium nitrite:* Bromhexin was given as a commercially available solution (2 mg/ml), and sodium nitrite was given in water (2 mg/ml). Female Wistar rats (four animals/group) were gavaged first with bromhexin (1-90 mg/kg) and then with sodium nitrite solution (1-90 mg/kg). Compounds were given as single doses (1-10 mg/kg) or as repeated doses (20-90 mg/kg; 10 mg/kg per h). Invariably, sodium nitrite was in a six-fold molar excess with respect to bromhexin. Ascorbic acid was administered in bromhexin solution, followed by sodium nitrite (10 mg/kg each) at molar ratios of ascorbic acid/nitrite between 2:1 to 1:2.

Urine samples were collected over a period of 32 h. Aliquots of the urine samples were heated to 90°C for 10 min. After cooling to room temperature, the urine was diluted with an equal volume of phosphate buffer (0.02 M, pH 6.5), and  $\beta$ -glucuronidase/arylsulfatase (0.2 ml) was added. After incubation for 48 h (46°C), samples were made alkaline with 2 N sodium hydroxide (0.5 ml), saturated with ammonium sulfate and applied to Extrelut®. After equilibration for 20 min, the columns were eluted with ethyl formate (50 ml), and the extracts were taken down to 1 ml by a nitrogen stream. Total OH-NMCA contents in urine were determined by GC-TEA; column: OV 351 6%, 3 m  $\times$  2.2 m, 230°C. Amounts were calculated from peak heights of control urine samples, spiked with 4-OH-NMCA.

*DNA methylation by NMCA in liver and oesophagus:* In order to investigate the in-vivo interaction of NMCA with DNA, single doses of [<sup>14</sup>C-methyl]-bromhexin (30 mg/kg; 49.5 mCi/mmol) and sodium nitrite (30 mg/kg) were administered orally to nine female Wistar rats. The animals were killed 4 h after treatment, organs were removed rapidly, frozen in liquid nitrogen and stored at -80°C. DNA from liver and oesophagus was isolated from the combined organs of all rats and purified as described elsewhere (Beland *et al.*, 1979). DNA was hydrolysed and analysed on Sephasorb HP columns (1.6  $\times$  50 cm) according to Hodgson *et al.* (1980). Radioactivity was determined in DNA adducts after addition of 10 ml Aqualuma® scintillation mixture. Amounts of methylated purines were expressed as fraction of guanine.

In a further experiment, ten female Wistar rats received a single intravenous injection of [<sup>14</sup>C-methyl]-NMCA (3 mg/kg; 42 mCi/mmol), and DNA from liver and oesophagus was isolated as described above.

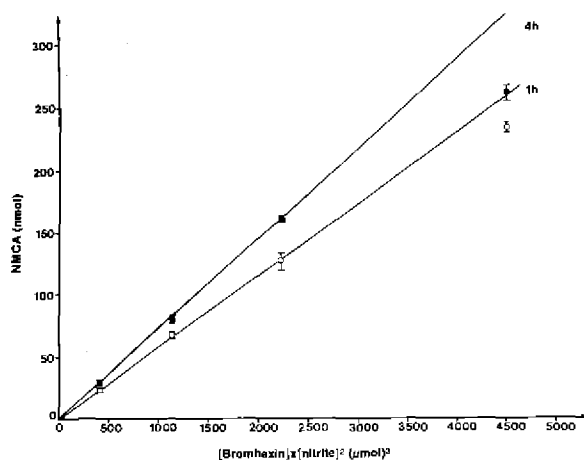
*Studies in humans:* Gastric juice of fasted (n=25) and pentagastrine-stimulated volunteers (n=21) was incubated *in vitro* with bromhexin (16 mg/100 ml) at 37°C for 1 h. Alternatively, gastric juice was taken from volunteers (n=29) 1 and 3 h after ingestion of up to 200 mg nitrate and incubated with bromhexin as described above. More recently, in another extensive study, volunteers ate vegetables corresponding to a nitrate intake of 200 mg. Gastric juice was taken 4 h later. Incubation with bromhexin was performed as described above.

## Results and discussion

The formation of an unidentified *N*-nitroso compound during the reaction of bromhexin and sodium nitrite under simulated gastric conditions was first detected by Scheunig and Ziebarth (1978). The compound formed under the conditions of the NAP has been identified by us as NMCA. After 1 and 4 h, NMCA was obtained in the range of 46-49% of the theoretical yield, irrespective of whether sulfamic acid or sodium hydroxide was used to quench the nitrosation after the allotted reaction times.

Reports on nitrosative cleavage of tertiary amines suggest that, at 37°C, highest yields of nitrosamines are observed at pH 3. Under NAP conditions, NMCA formation is comparable to the formation of *N*-nitrosodimethylamine from Aminophenazon and sodium nitrite (Lijinsky, 1974; Gillatt *et al.*, 1984). In the low micromolar range, however, yields of NMCA were considerably lower and varied from 0.15-2.7% of the theoretical yield. A direct dependence on the square of the nitrite concentration can be seen (Fig. 1).

**Fig. 1. Effect of sodium nitrite concentration on in-vitro formation of NMCA after 1 (○) and 4 (●) h**



Bromhexin (kept constant at 9.7 μmol) was incubated with sodium nitrite (2.2-22 μmol) in citrate/hydrochloric acid buffer pH 3. Amounts of NMCA are plotted as a function of [Bromhexin] × [Nitrite]<sup>2</sup>.

sulfate conjugates and 8-18% as free metabolites. A linear dose-related increase in the excretion of OH-NMCA in the 32-h urine following administration of the precursors can be seen (Fig. 2).

Co-administration of ascorbic acid together with bromhexin and sodium nitrite at molar ratios of ascorbic acid:nitrite between 2:1 and 1:2 resulted in a reduction of the urinary OH-NMCA levels down to ≤ 5% in all cases. This demonstrates that ascorbic acid inhibits NMCA formation in rats by ≥ 95%, even when given in a molar deficit to nitrite.

To confirm the results of Schlag *et al.* (1980), who found a linear correlation between log nitrite concentration and pH in gastric juice, the dependence of NMCA formation on pH (range, 2-7) and the corresponding mean nitrite levels (0.4-50 μg/ml) was investigated in a further experiment. Table 1 shows a comparison of yields obtained in buffer and in gastric juice. It can be seen that, in gastric juice, nitrosation is detectable only at pH 3, and yields in gastric juice are lower than in buffer.

When bromhexin (1-90 mg/kg) was given intragastrically together with sodium nitrite (1-90 mg/kg), ring-hydroxylated metabolites were detected in urine at doses down to 1 mg/kg of bromhexin and sodium nitrite, respectively. Throughout the dose range investigated, 82-92% of the OH-NMCA determined was excreted as glucuronide-

**Table 1. Formation of NMCA after incubation (1 h, 37°C) of 16 mg/100 ml<sup>a</sup> bromhexin in buffer or gastric juice with adjusted pH and nitrite content**

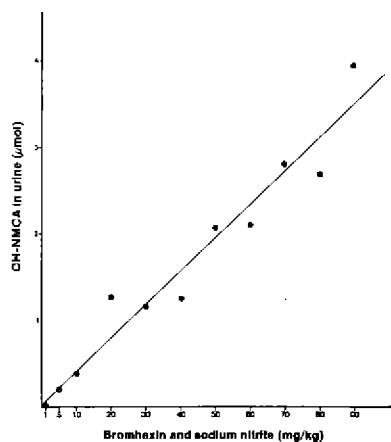
pH	Nitrite ( $\mu\text{g}/\text{ml}$ ) <sup>b</sup>	NMCA (ng/ml) <sup>a</sup>	
		Buffer	Gastric juice
2	0.4	ND	ND
3	0.8	ND	ND
	2.5	2.5	2
	6.5	29	16.4
4	2.5	0.9	ND
	4.5	3.4	ND
5	6.5	ND	ND
6	20.0	ND	ND
7	50.0	ND	ND

<sup>a</sup>100 ml, presumed volume of a fasted stomach

<sup>b</sup>Mean nitrite level at each pH corresponds to 1.

ND, not detectable at < 0.5 ng/ml

**Fig. 2. Excretion of OH-NMCA in urine versus doses of bromhexin/sodium nitrite**



Bromhexin and sodium nitrite (1-90 mg/kg each) were given by gavage to groups of four fasted rats; ●, mean value of the determination of three aliquots; —, linear regression fit of the means

Lijinsky *et al.* (1973) reported on the examination of liver and oesophagus nucleic acids after oral administration of a mixture of [ $d_3$ -methyl]-NMCA and [ $^3\text{H}$ -cyclohexyl]-NMCA to rats. At 16 h after treatment, the authors found deuterium-labelled *N7*-methylguanine in liver RNA and DNA, but not in oesophageal RNA or DNA; they also found no evidence for the formation of tritium-labelled adducts.

In the present investigation, the extent of DNA methylation in liver and oesophagus was determined 4 h after intravenous injection of 3 mg/kg [ $^{14}\text{C}$ -methyl]NMCA and after oral administration of 30 mg/kg each of [ $^{14}\text{C}$ -methyl]-bromhexin and sodium nitrite, respectively. In both experiments, the greatest extent of methylation was found in the oesophagus, indicating that preferential alkylation is independent of the route of administration.

Table 2 shows that after administration of [ $^{14}\text{C}$ -methyl]-NMCA, concentrations of methylated guanines in oesophagus were 13 times (*N7*-methylguanine) and 12 times (*O6*-methylguanine) higher than in liver. When the precursors were given, the respective ratios of oesophagus to liver methylation were 5:1 (*N7*-methylguanine) and 8:1 (*O6*-methylguanine). Our results suggest that induction of oesophageal tumours by NMCA correlates well with the preferential alkylation of DNA in the target tissue.

Results of the in-vivo/in-vitro studies (see above) indicate that NMCA was formed in the gastric juice of only one volunteer (1 h after ingestion of 100 mg nitrate), at a concentration of 50 ng/100 ml. Further results will be reported.

Yields of NMCA differ considerably under the conditions of the NAP and in experiments with low concentrations of precursors in buffer or gastric juice. Reasons that only trace amounts of NMCA were found under physiological conditions are: (i) in gastric juice, bromhexin is in competition with

**Table 2. Methylated purines in DNA of liver and oesophagus following a single dose of [<sup>14</sup>C-methyl]-NMCA<sup>a</sup> or [<sup>14</sup>C-methyl]-bromhexin<sup>b</sup> and sodium nitrite**

Treatment	Methylated purines <sup>c</sup>					
	Liver			Oesophagus		
	<i>N7</i> -meG	<i>O6</i> -meG	<i>O6:N7</i>	<i>N7</i> -meG	<i>O6</i> -meG	<i>O6:N7</i>
[ <sup>14</sup> C-me]-NMCA	2.3	0.3	0.11	27.7	3.9	0.14
[ <sup>14</sup> C-me]-Bromhexin	2.3	0.15	0.08	10.3	1.2	0.12

<sup>a</sup>4 h after a single intravenous injection of [<sup>14</sup>C-methyl]-NMCA (3 mg/kg)

<sup>b</sup>4 h after intragastric administration of [<sup>14</sup>C-methyl]-bromhexin and sodium nitrite (30 mg/kg each)

<sup>c</sup>Expressed as fraction of guanine × 10<sup>6</sup>

other compounds for available nitrite; (ii) the formation of NMCA depends on the square of nitrite concentration and occurs at measurable yields only in a narrow pH range, with an optimum at pH 3; and (iii) the pH of gastric juice and nitrite concentration are correlated; this results in negligible formation of NMCA at high nitrite concentrations, since the pH is unfavourable; at pH 3 or lower, nitrite concentrations are apparently insufficient to generate NMCA in measurable yields.

#### Acknowledgements

We thank the Institute of Biochemistry, Dr Karl Thomae GmbH, Biberach/Riss, for valuable cooperation.

# THE ROLE OF VARIOUS FOOD PRODUCTS IN THE FORMATION OF *N*-NITROSO COMPOUNDS UNDER ACIDIC CONDITIONS

L.W. van Broekhoven<sup>1</sup>, W.M.F. Jongen<sup>2</sup>, J.A.R. Davies<sup>1</sup>  
& M.A.J.S. van Boekel<sup>3</sup>

<sup>1</sup>Centre for Agrobiological Research, PO Box 14, 6700 AA Wageningen;

<sup>2</sup>Department of Toxicology and <sup>3</sup>Department of Food Science,  
Agricultural University, De Dreyen 12, 6703 BC Wageningen, The Netherlands

The effect of lettuce cultivars on the nitrosation rate of proline was investigated. The lettuce was analysed for the presence of phenolic compounds. Lettuce and/or fish was incubated with nitrite under acidic conditions, and the incubation mixtures were investigated for the presence of *N*-nitroso compounds and mutagenic activity. Both volatile *N*-nitrosamines and mutagenic nonvolatile *N*-nitrosamines were detected. The formation of mutagenic *N*-nitroso compounds was also studied in selected cheese products after treatment with nitrite under acidic conditions. No direct relationship was observed between the total *N*-nitroso content of the samples and the corresponding mutagenicity. The ability of cheese to inhibit the direct mutagenicity occurring in fava beans after treatment with nitrite under acidic conditions was investigated. The antimutagenic factor, possibly casein, in cheese was not extractable with different solvents.

Interactions between different food products with respect to the formation of mutagenic *N*-nitroso compounds after treatment with nitrite under acidic conditions were studied. Special attention was paid to the formation of nonvolatile *N*-nitrosamines.

## Influence of lettuce on the nitrosation of proline

Eight different lettuce cultivars (*Lactuca sativa* L.) were investigated for their potential to influence the nitrosation of proline with nitrite at pH 3. Lyophilized samples were analysed for the presence of constituents able to affect the nitrosation rate (Table 1).

The lettuce samples (1 g) were added to an incubation medium (100 ml) containing 1 mmol/l of proline and nitrite. Nitrosation was performed at pH 3 and 37°C for 0.25 h; the reaction was stopped with excess ammonium sulfamate. *N*-Nitrosoproline (recovery, 85%) was extracted and determined as described previously (van Broekhoven *et al.*, 1984).

All cultivars showed an inhibition of 21-36%. Under these conditions, an inhibition of 90% was found with 1 mmol/l chlorogenic acid; no effect was found for amounts corresponding to those found in lettuce (~ 10 µmol/l). Varietal differences were not significant with respect to inhibition of the formation of *N*-nitrosoproline. The diminution of the nitrosation rate cannot, therefore, be explained by the amount of phenolic compounds present.

**Table 1. Presence of compounds able to influence nitrosation rate in eight different lettuce cultivars**

Cultivar <sup>a</sup>	Dry matter (g/kg fresh)	Chlorogenic acid (mg/kg dry matter)	Dicaffeoyl tartaric acid (mg/kg dry matter)	Ascorbic acid (mg/kg dry matter)	Nitrate (g N/kg dry matter)	Total nitrogen (g/kg dry matter)
1	69.7	70	330	16	16	59
2	57.4	80	320	33	17	59
3	74.5	120	260	27	13	60
4	64.9	50	310	19	15	59
5	75.1	80	300	19	9	52
6	91.1	40	120	94	11	57
7	65.1	40	130	22	11	54
8	80.3	40	170	23	9	53

<sup>a</sup>Obtained from the Institute for Horticultural Plant Breeding, Wageningen

#### Formation of N-nitroso compounds from lettuce and/or fish under 'simulated gastric' conditions

Lettuce (0.5 g) and/or fish (1 g) (both lyophilized) was reacted with nitrite under acidic ('simulated gastric') conditions (150 ml of a 7 g/l solution of sodium chloride; 0.75 g pepsin; 300 mg sodium nitrite; pH 3.4; 0.5 h; 37°C); the reaction was stopped with excess ammonium sulfamate. The incubation mixture was extracted with dichloromethane (volatile N-nitrosamines) and with ethyl acetate at pH 2 and pH 11 (nonvolatile N-nitrosamines). The presence of volatile N-nitrosamines was determined by gas chromatography/thermal energy analysis (Table 2). The ethyl acetate fractions were analysed for the presence of nonvolatile N-nitrosamines by total N-nitroso determination (method of Walters *et al.*, 1980; Table 2). These fractions were also analysed with a photohydrolysis detector (PHD) after separation by high-performance liquid chromatography (HPLC; Shuker & Tannenbaum, 1983). (Stationary phase: Radial-Pak C-18 cartridge (Waters Assoc., Milford, MA, USA); mobile phase: 30 mM monobasic ammonium phosphate buffer pH 6:acetonitrile 20:1; flow: 0.5 ml/min.) All samples were also analysed by HPLC/PHD with the lamp turned off, to detect false negatives.

**Table 2. N-Nitroso content of samples after incubation under 'simulated gastric' conditions**

Sample	N-Nitroso-dimethylamine (µg/l)	Total N-nitroso content <sup>a</sup> (µmol/l)	
		pH2	pH 11
Lettuce	ND <sup>b</sup>	6	2
Lettuce and fish	117	15	2
Fish	107	11	2

<sup>a</sup>No residual nitrite was detected.

ND, not detected (< 3 µg/l)

The pH 2 fractions gave a badly separated set of peaks; the pH 11 fractions gave only one peak at the front. The positive samples were separated by HPLC into two fractions ( $0 < R_t < 19$  min and  $19 \text{ min} < R_t < 28$  min) and tested for mutagenic activity in a modified *Salmonella*/mammalian microsome assay after metabolic activation (Table 3).

**Table 3. Mutagenic activity in *Salmonella typhimurium* strain TA100<sup>a</sup> of samples after incubation with nitrite under 'simulated gastric' conditions**

Sample	A	B	C	D
	(revertants/plate <sup>b</sup> )			
Control	138	149	140	147
Lettuce	237	184	172	238
Lettuce and fish	804	441	315	517
Fish	868	463	351	NT

<sup>a</sup>*Salmonella*/mammalian microsome assay: preincubation time, 2 h; S9 mix prepared in phosphate buffer pH 6 containing 4% rat liver S9 and 5 mM calcium chloride

<sup>b</sup>Control value, 132 rev/plate; A, ethyl acetate (pH 2); B, A (1); C, A (2); D, ethyl acetate (pH 11); NT, not tested. One plate corresponds to 39 ml incubation mixture containing 0.13 g lettuce and/or 0.26 g fish.

The results indicate the presence of nonvolatile *N*-nitrosamines in the incubation medium. The peaks found by HPLC/PHD from the incubation with lettuce were the same as those in the blank, although they disappeared when the lamp was turned off. These samples showed no or only low mutagenic activity. Only the incubations with lettuce plus fish and those with fish alone showed the presence of peaks different from the blank and were mutagenic. Lettuce thus has no effect on the formation of mutagenic *N*-nitrosamines from fish under these conditions. Only the nitrate content of lettuce seems important for *in vivo* formation.

#### Dual role of cheese in the formation of *N*-nitrosamines during treatment of food constituents with nitrite

Various cheese products (lyophilized, defatted) were incubated under 'simulated gastric' conditions (see above). The incubation mixtures were extracted with dichloromethane and ethyl acetate (pH 2 and pH 11). The results are shown in Table 4. No direct relationship was observed between the total *N*-nitroso content of the samples and the mutagenicity. Different cheese products must contain different types of indirectly acting *N*-nitroso compounds. In processed cheese, potential interference (possibly Maillard products?) might be introduced by the heat treatment used in its preparation.

**Table 4. Mutagenicity and amounts of *N*-nitroso compounds in different cheese products after incubation with nitrite under 'simulated gastric' conditions**

Product	A		B		C	
	rev/g	μmol/g	rev/g	μmol/g	rev/g	μmol/g
Gouda young	1224	0.12	660	0.14	96	0.01
Gouda old	1672	0.10	1664	1.80	684	0.07
Processed cheese	1660	0.21	9	0.25	3874	0.01
Camembert	396	0.07	256	0.25	784	0.04
Cheddar	672	0.05	NT	0.24	NT	0.04
Limburger	256	0.12	168	0.88	196	0.12

<sup>a</sup>Control value (143 rev/plate) has been subtracted; A, dichloromethane; B, ethyl acetate (pH 2); C, ethyl acetate (pH 11); NT, not tested

Lyophilized defatted cheese inhibited the directly-acting mutagens found in fava beans after incubation with nitrite under 'simulated gastric' conditions (see above). After addition of increasing amounts of cheese to the filtered incubation mixture, a dose-related decrease in the number of revertants was observed (Fig. 1). The residual amount of *N*-nitroso compounds parallels the decrease in mutagenicity, indicating that the antimutagenic effects observed are the consequence of a reduction in the content of *N*-nitroso compounds.

**Fig. 1. Inhibitory effect of defatted cheese on mutagenicity generated in fava beans after treatment with nitrite under acidic conditions**

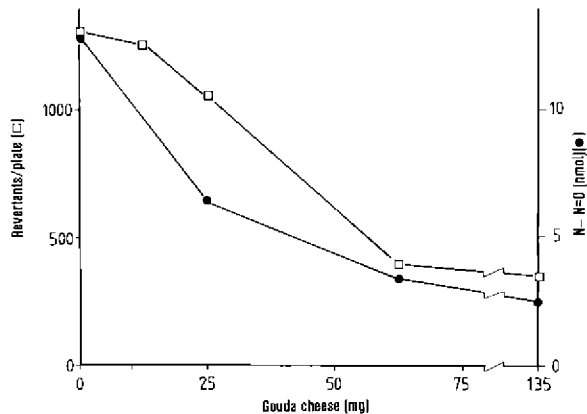


Table 5 shows the results of successive extractions of cheese with solvents of increasing polarity. Obviously, the modulating factor is not extractable and is likely to be a protein. This was confirmed by model experiments in which casein (the dominant protein in cheese) and other macromolecular food constituents showed antimutagenic activity.

**Table 5. Effects of successive Soxhlet extractions with different solvents on the inhibitory potential of cheese residues on the mutagenicity of *N*-nitroso compounds in 62.5 mg fava beans after treatment with nitrite**

Treatment	Extraction solvent	Residue weight (mg)	Strain TA100 (revertants/plate) <sup>a</sup>	Total <i>N</i> -nitroso-compounds (nmol)	Inhibition (%)
No cheese	-	-	1041	8.2	0
Cheese	-	125.0	653	3.9	44
Extracted cheese	light petroleum	62.5	265	2.1	88 <sup>b</sup>
	+ chloroform	62.5	247	2.3	90
	+ methanol	51.2	238	2.0	91
	+ water	38.8	236	2.1	91
Casein	-	62.5	266	1.9	88

<sup>a</sup>Control value, 162 revertants/plate

<sup>b</sup>Effect of increased solubility of cheese in water

#### Acknowledgements

The valuable contributions of B. de Bruin, M.C. Jansen and P.J. Koole to this study are gratefully acknowledged.



# ACTIVATION OF *N*-NITROSODIALKYLAMINES BY NEAR-ULTRAVIOLET IRRADIATION: FORMATION OF DIRECTLY-ACTING MUTAGENS AND DNA-DAMAGING PRODUCTS

H. Shimada<sup>1</sup>, K. Yakushi<sup>1</sup>, A. Ikarashi<sup>2</sup>, M. Mochizuki<sup>2</sup>, E. Suzuki<sup>3</sup>, M. Okada<sup>3</sup>, S. Yokoyama<sup>4</sup>, T. Miyazawa<sup>4</sup> & H. Hayatsu<sup>1</sup>

<sup>1</sup>Faculty of Pharmaceutical Sciences, Okayama University, Tsushima, Okayama; <sup>2</sup>Kyoritsu College of Pharmacy, Tokyo; <sup>3</sup>Tokyo Biochemical Research Institute, Tokyo; and <sup>4</sup>Faculty of Science, University of Tokyo, Tokyo, Japan

On near-ultraviolet (UVA) irradiation in a phosphate buffer, *N*-nitrosomorpholine (NMOR) and *N*-nitrosopyrrolidine (NPYR) were converted into directly-acting mutagens. The activated NPYR was fractionated, and the active product was isolated. The compound was shown to be identical to  $\alpha$ -phosphonoxy NPYR on the basis of several properties: retention times in high-performance liquid chromatograms, mutagenic specificity and potency, ultraviolet spectrum, and inactivation by phosphatase treatment. Photoactivation was inhibited by superoxide dismutase, and therefore superoxide is implicated as playing a key role in mutagen formation. *N*-Nitrosoproline (NPRO) and eighteen other *N*-nitrosodialkylamines were irradiated with UVA in the presence of  $\phi$ X174 RFI DNA. The DNA underwent single-strand breaks to give RFII DNA, indicating that *N*-nitrosodialkylamines in general have this property. DNA chain cleavage was inhibited both by superoxide dismutase and hydroxyl-radical scavengers. These results provide new information on the genotoxic mechanism of action of *N*-nitrosodialkylamines.

We have found that some *N*-nitrosodialkylamines can be activated to directly-acting mutagens by UVA irradiation (Hayatsu *et al.*, 1984; Shimada & Hayatsu, 1985). Furthermore, most irradiated *N*-nitrosodialkylamines give rise to products that can cause DNA single-strand breaks. Clarification of the mechanism of these reactions may reveal new aspects of the genotoxic action of *N*-nitrosodialkylamines.

## Mutagenicity arising from UVA irradiation of *N*-nitrosodialkylamines

As reported earlier (Hayatsu *et al.*, 1984), *N*-nitroso alicyclic amines, e.g., NMOR, NPYR and *N*-nitrosopiperidine (NPIP), generated directly-acting mutagens when aqueous solutions containing them, with phosphate or phosphate esters, were irradiated with UVA. The ability of UVA to activate these nitrosamines is stronger than that of S9 (Table 1. Such photoactivation is dependent on the presence of phosphate or its esters. In paper chromatography of the reaction products, the migration distance of the directly-acting mutagen(s) formed from NMOR was a function of the polarity of the phosphates used — the more polar the phosphate compound, the smaller the R value of the mutagenic product (Hayatsu *et al.*, 1984).

**Table 1. Mutagenicity of *N*-nitrosamines towards *S. typhimurium* TA100<sup>a</sup>**

<i>N</i> -Nitrosamine	No. of <i>his</i> <sup>+</sup> revertants/5 $\mu$ mol	
	S9 <sup>b</sup>	UVA <sup>c</sup>
NMOR	332	7271
NPYR	144	636
NPIP	99	540

<sup>a</sup>Averages of duplicate experiments, found by subtracting results for solvent controls (100-140) from the observed numbers of revertants colonies

<sup>b</sup>Livers of phenobarbital-induced rats used in a preincubation assay (Yahagi *et al.*, 1977) at 37°C for 40 min

<sup>c</sup>Reaction mixture containing 40 mM *N*-nitrosamine and 20 mM sodium phosphate, pH 7.4, was irradiated with  $9.7 \times 10^4$  J/m<sup>2</sup> UVA; then, aliquots were taken and assayed for mutagenicity without addition of S9

This directly-acting mutagen was identical in the following properties with  $\alpha$ -phosphonoxy NPYR, which had been synthesized by hydrolysis of  $\alpha$ -acetoxy NPYR in a neutral inorganic phosphate buffer: (1) identical retention times in HPLC on both the reverse-phase column and an ion-exchange column (Partisil 10 SAX, Sumitomo); co-injections were done; (2) identical sensitivity to phosphatase: both our sample and  $\alpha$ -phosphonoxy NPYR were inactivated by treatment with bacterial alkaline phosphatase; (3) identical ultraviolet absorption spectra in an aqueous solution at pH 7, giving an absorption maximum at 229 nm; (4) similar specificity and potency in mutagenicity to *S. typhimurium* and *Escherichia coli* WP2: they were strong mutagens towards TA1535 and TA100, and weaker mutagens towards TA102; both had a mutagenic potency to TA1535 in the absence of S9 of about  $5 \times 10^4$  revertants per A<sub>229</sub> nm unit.

Sulfhydryl compounds such as cysteine, cysteamine and glutathione were found to inhibit the photoactivation of NMOR strongly. Since superoxide dismutase was also an effective inhibitor, the activation must require the presence of oxygen free-radicals. Hydroxyl radical ( $\cdot$ OH) scavengers such as ethanol and benzoic acid did not inhibit the UVA-induced activation.

#### DNA single-strand breaks induced by UVA irradiation in the presence of *N*-nitrosodialkylamines

The breaks were detected by irradiating phage  $\phi$ X174 replicative form DNA (RFI) with UVA in the presence of 2 mM nitrosamine (at pH 7.4 and room temperature, for 1 h) and then analysing the DNA by agarose-gel electrophoresis. Single-strand breaks give rise to the formation of the relaxed circular form RFII, which migrates in the gel more slowly than RFI. Details of this experimental system have already been described (Wakata *et al.*, 1985). Single-strand breaks were induced by all of 19 *N*-nitrosodialkylamines examined: *N*-nitrosodimethylamine, *N*-nitrosodiethylamine, *N*-nitrosodi-*n*-propylamine, *N*-nitrosoethyl-*n*-butylamine, *N*-nitroso-*n*-propyl-*n*-butylamine, NMOR, NPYR, NPIP, NPRO, *N*-nitrosoethyl(2-hydroxyethyl)amine, *N*-nitroso-*n*-butyl(2-hydroxyethyl)amine

We have isolated the directly-acting mutagen formed from NPYR and inorganic phosphate. After NPYR was irradiated with UVA (Hayatsu *et al.*, 1984), the reaction mixture was washed with chloroform to remove unreacted NPYR, and then mixed with ethanol to precipitate inorganic phosphate. The soluble fraction was subjected to paper chromatography using *n*-propanol:concentrated ammonium hydroxide:water (55:10:35) as the solvent system, and the extracted fraction was analysed by high-performance liquid chromatography (HPLC) on a reverse-phase column ( $\mu$ -Bondapak C<sub>18</sub>, Sumitomo, Japan). The column was eluted with 20 mM ammonium phosphate at pH 7, and the active fraction was obtained as a single peak (detection at A<sub>254</sub> nm).

(NBHEA), *N*-nitrosodiethanolamine, *N*-nitrosoethyl(3-hydroxypropyl)amine, *N*-nitroso-*n*-butyl(3-hydroxypropyl)amine, *N*-nitrosoethyl(4-hydroxybutyl)amine, *N*-nitroso-*n*-butyl(4-hydroxybutyl)amine (NBHBA), *N*-nitroso-*n*-butylcarboxymethylamine, *N*-nitroso-*n*-butyl(2-carboxyethyl)amine and *N*-nitroso-*n*-butyl(3-carboxypropyl)amine (NBCA).

DNA chain cleavage was dependent on both the nitrosamine and UVA. *N*-Nitrosodialkylamines with a hydroxyl or a carboxyl group in the alkyl chain, e.g., NBHEA, NBHBA and NBCPA, had particularly strong activity. In contrast, the *N*-nitramines corresponding to these three *N*-nitrosamines induced no DNA break, indicating that the nitroso function is essential for this reaction. It is noteworthy that NPRO can cleave DNA on irradiation. NPRO has been reported to be nonmutagenic and noncarcinogenic (Mirvish *et al.*, 1980a). L-Proline is often administered to humans for measuring urinary levels of NPRO as an indicator of endogenous nitrosation (Lu *et al.*, 1986).

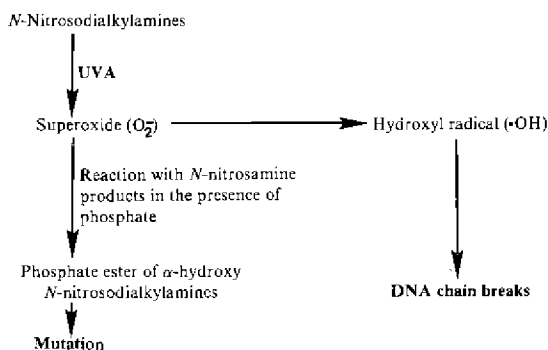
Our results show that the ability to break DNA with UVA irradiation may be a general property of *N*-nitrosamines. It should be noted that this activity of *N*-nitrosamines does not require phosphate; therefore, the DNA-breaking effect is distinct from the formation of directly-acting mutagens by UVA irradiation of *N*-nitrosamines.

Formation of DNA breaks was inhibited by sulfhydryl compounds and superoxide dismutase. Addition of hydroxyl radical scavengers such as ethanol, benzoic acid and D-mannitol to the reaction mixture also inhibited DNA breakage. These results suggest that hydroxyl radicals are involved in this reaction. Hydroxyl radicals are well-known DNA-damaging agents, causing single-strand breaks (see, for example, Janicek *et al.*, 1985).

### Mechanism of photoactivation

These two distinct activities of UVA-irradiated *N*-nitrosodialkylamines—direct mutagenicity and DNA cleavage—appear to occur through the formation of active oxygen radicals (Fig. 1). Both mutagenesis and DNA breakage were inhibited by superoxide dismutase; the mutagenesis was not suppressed by hydroxyl radical scavengers, while the chain breaks were efficiently inhibited by these scavengers. The  $\alpha$ -hydroxylated form of an *N*-nitrosodialkylamine has been considered to be the active principle in the mutagenic action of metabolically altered nitrosamines. It is likely that the phosphate ester of the  $\alpha$ -hydroxyl derivative is dephosphorylated before it can exert its mutagenicity. It would be important to examine the possibility that these phosphate esters can be generated *in vivo*, without light, as metabolites of *N*-nitrosodialkylamines (see Frank & Wiessler, 1986b, for such possibilities). A recent report by Sterzel and Eisenbrand (1986) suggests that the sulfate conjugate of  $\beta$ -hydroxylated *N*-nitrosodiethanolamine metabolites can cause single-strand breaks in DNA.

Fig. 1. Mechanism of photo-activation



# OBSERVATIONS ON THE USE OF <sup>137</sup>CAESIUM RADIATION TO CONTROL *N*-NITROSOPYRROLIDINE FORMATION IN BACON

W. Fiddler, J.W. Pensabene, R.A. Gates & R.K. Jenkins

*US Department of Agriculture, Agricultural Research Service,  
Eastern Regional Research Center, 600 E. Mermaid Lane,  
Philadelphia, PA 19118, USA*

Several alternatives to the use of nitrite, including irradiation, have been developed to reduce the nitrosamine content in bacon and still retain its microbiological safety and desirable sensory characteristics. This paper presents results obtained from experiments in which <sup>137</sup>Cs was used at +5°C. Bacon prepared with 120 or 40 mg/kg sodium nitrite (NaNO<sub>2</sub>) yielded lower residual nitrite before frying and lower levels of *N*-nitrosopyrrolidine (NPYR) after frying when irradiated at 3.0 Mrad, compared to doses of 0, 0.75 and 1.50 Mrad. Also, a slight increase in the level of NPYR in fried bacon over the 0 control was noted with 0.75 Mrad. In bacon irradiated with 0-1.5 Mrad in 0.25-Mrad increments, a marked increase was observed at 0.5 Mrad. Bacon from pork bellies irradiated prior to processing had more NPYR after frying than bacon irradiated after processing, suggesting the formation of an additional precursor or some catalytic agent.

Sterilization by radiation has been proposed as a possible means of reducing levels of nitrite in bacon to the minimum needed for the development of the characteristic cure colour and flavour, and also to provide the requisite protection against *Clostridium botulinum*. Most of the earlier research on the irradiation of bacon was carried out at a single dose of 3.0 Mrad with a <sup>60</sup>Co isotope source at -40°C (Fiddler *et al.*, 1981). The object of the present study was to determine if <sup>137</sup>Cs would give similar results with regard to NaNO<sub>2</sub>/NPYR values to those obtained with <sup>60</sup>Co.

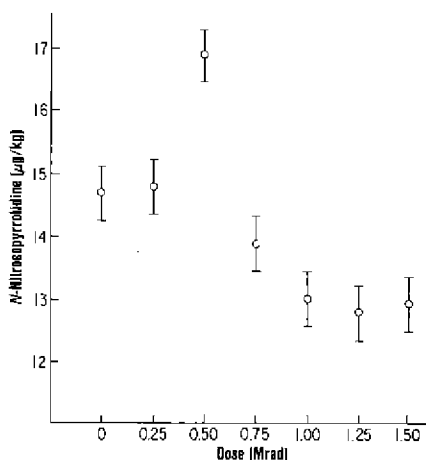
We initially conducted studies in which bacon, prepared with 120 or 40 mg/kg NaNO<sub>2</sub> and with and without 550 mg/kg sodium ascorbate, was subjected to <sup>137</sup>Cs irradiation (~ 0.01 Mrad/min) at 5°C (Pensabene *et al.*, 1986). The irradiation conditions and dosimetry have been described by Shieh *et al.* (1985). The 3.0-Mrad dose was found to yield lower residual nitrite in bacon before frying, and lower NPYR after frying, at both nitrite levels, when compared to the 0, 0.75 and 1.5 Mrad irradiated samples. The decrease (67%) in added NaNO<sub>2</sub>, from 120 to 40 mg/kg, was reflected in lower NPYR values (61-67%) in the 40 mg/kg NaNO<sub>2</sub> fried product at all dose levels. In addition, lower overall NPYR values were obtained in bacon prepared with 120 mg/kg NaNO<sub>2</sub> with 550 mg/kg ascorbate compared to bacon with no ascorbate added.

## Apparent enhancement of NPYR

An apparently higher NPYR content was observed in some bacon prepared with 120 mg/kg NaNO<sub>2</sub> and irradiated at 0.75 Mrad. To determine whether this observation was real,

we conducted experiments with bacon irradiated with 0-1.5 Mrad in 0.25-Mrad increments. Fried bacon from 12 of 16 individual bellies showed an elevation of at least  $1.5 \mu\text{g}/\text{kg}$  NPYR between 0.25 and 0.75 Mrad (Fig. 1). The elevated NPYR averaged  $2\text{-}3 \mu\text{g}/\text{kg}$ , probably had no toxicological significance, and was centred at the 0.5 Mrad dose level. This increase in NPYR content also appeared in nine of 12 samples of cooked-out drippings from the corresponding bacon. There is evidence that  $\gamma$ -radiation catalyses the formation of *N*-nitrosopiperidine in a model system (Challis *et al.*, 1980). An increase at lower dose levels and subsequent decrease at higher doses could be explained by destruction of the nitrosamine, once formed. However, this would not be the case with regard to bacon, because NPYR is formed at the time of frying. It was therefore theorized that in the 0.25 and 0.75 Mrad dose range some compounds might be formed that would catalyse nitrosation at the time of frying and that might be destroyed at higher dose levels.

**Fig. 1. NPYR in fried bacon irradiated at different dose levels**



Overall mean and 95% confidence interval;  $n = 12$

6.66 and  $5.30 \mu\text{g}/\text{kg}$ , respectively, with a reproducibility of  $0.52 \mu\text{g}/\text{kg}$ . Under the experimental conditions employed, the amount of nitrosamine precursor-catalyst should be the same, the only differences being the residual nitrite (which correlates well with NPYR). The average levels of residual  $\text{NaNO}_2$  in the pre- and post-processed irradiated bacon were 73.8 and  $66.4 \text{ mg}/\text{kg}$ , respectively.

The increase in NPYR levels in the 0.25-0.75 Mrad dose range may have been due to formation of a catalytic agent through the secondary lipid peroxidation product, malonaldehyde, which has been shown to enhance the nitrosation of dimethylamine in a model system at a pH comparable to that of meat (Kikugawa *et al.*, 1985). This compound is highly reactive and may not be present at higher dose levels. The lipid hydroperoxide of 2-oleodistearin has also been found to stimulate the formation of NPYR in a methanolic reaction mixture with  $\text{NaNO}_2$  and proline (Coleman, 1978). The formation of lipid

To study this apparent increase in NPYR and to preclude the possibility of forming a more efficient nitrosating species as a result of  $\gamma$ -radiation, one set of seven matched pairs of pork bellies was irradiated (0-1.5 Mrad in 0.25 increments) prior to curing with nitrite and processing into bacon. The other matching pairs of pork bellies were pumped with a cure mixture, so that  $120 \text{ mg}/\text{kg}$  sodium nitrite and no ascorbate were added; they were then treated thermally and smoked, like the other pair. This bacon was irradiated under the same conditions as the green pork bellies. All the bacon was fried under the same conditions ( $177^\circ\text{C}$  for 3 min/side). Only four matched pairs were used for analysis of variance because they exhibited an increase of greater than  $1 \mu\text{g}/\text{kg}$  NPYR (twice the reproducibility). Irradiation of the bellies prior to processing gave small, but significantly higher NPYR levels in the edible fried bacon than those obtained after bacon processing: average values were

hydroperoxide, however, would be expected to reduce NPYR formation in a manner analagous to the inhibition of nitrosation of dimethylamine, due to the reaction of the hydroperoxide with nitrite to form nitrate, as reported by Kikugawa *et al.* (1985).

Clearly, the role of lipid oxidation products in NPYR production may be a complex one. Should the recent finding of Tricker *et al.* (1985) prove true — that free proline in adipose tissue is a major precursor of NPYR —  $\gamma$ -induced decarboxylation of proline may occur at the lower dose levels indicated. Spinelli-Gugger *et al.* (1981) indicated that a proline-free peptide fraction derived from bacon adipose tissue was responsible for nearly all the NPYR formed. A study of the mechanism of NPYR formation and its precursors is required in order to elucidate the direct effect of irradiation. Current investigations are therefore focusing on the breakdown of collagen in bacon adipose tissue. The present results also indicate that <sup>137</sup>Cs can be effective in reducing higher NPYR levels in fried bacon.

# PREVENTION OF EXPOSURE TO *N*-NITROSAMINES IN THE RUBBER INDUSTRY: NEW VULCANIZATION ACCELERATORS BASED ON 'SAFE' AMINES

C.-D. Wacker<sup>1</sup>, B. Spiegelhalder<sup>1</sup>, M. Börzsönyi<sup>2</sup>, G. Brune<sup>1</sup> & R. Preussmann<sup>1</sup>

<sup>1</sup>*Institute of Toxicology and Chemotherapy, German Cancer Research Centre,  
Im Neuenheimer Feld 280, D-6900 Heidelberg 1, Federal Republic of Germany;*  
and <sup>2</sup>*National Institute of Hygiene, Budapest, Hungary*

Introduction of 'safe' amino components into traditional accelerator molecules could be an effective measure to prevent formation of carcinogenic *N*-nitroso compounds during rubber production. About 20 new derivatives of the dithiocarbamate and sulfenamide class, based on 'safe' amines, were synthesized and shown to be suitable for industrial application. Some of the corresponding *N*-nitrosamines were prepared and investigated for mutagenicity in *Salmonella typhimurium* TA1535. No or weak mutagenic potential was observed in most cases. The nitrosatability of five sulfenamides derived from 'safe' amines was determined and found to be substantially lower than that of a commercial sulfenamide accelerator tested under identical conditions.

Occupational exposure to nitrosamines is still a major, unsolved problem in the rubber and tyre industry (Spiegelhalder & Preussmann, 1982). Appreciable amounts of *N*-nitrosodimethylamine (NDMA), *N*-nitrosodiethylamine, *N*-nitrosodibutylamine, *N*-nitrosopiperidine and *N*-nitrosomorpholine (NMOR) were detected in all indoor workrooms of 19 factories in the Federal Republic of Germany investigated between 1980 and 1983 (Spiegelhalder & Preussmann, 1983a). It is our aim to find preventive measures to reduce exogenous exposure to these nitrosamines.

## Concept of 'safe' amines

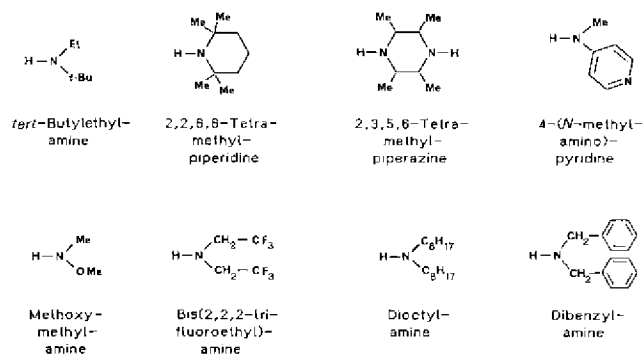
Previous studies (summarized by Fishbein, 1985) showed that the occurrence of carcinogenic *N*-nitrosamines in the rubber industry can be related to the use of certain vulcanization accelerators and stabilizers, such as dithiocarbamates, thiurams and sulfenamides. Since it seems impossible to avoid nitrosation of these accelerators and/or their thermal degradation products, it is necessary to look for alternative curing systems that do not form carcinogenic *N*-nitroso compounds during (or after) the production process.

The best method for avoiding the formation of *N*-nitrosamines might be the introduction of nitrogen-free accelerators. Unfortunately, only a few chemical classes are known, e.g., organic peroxides, of which the derivatives fulfil this criterion and are suitable for the vulcanization of rubber mixtures. The use of nitrogen-free accelerators is limited to some special applications.

In this situation, our concept of 'safe' amines could provide a solution. It is known that several types of secondary amines, e.g., dibenzylamine, alkyl-*tert*-butylamines and some

methylated piperidines and piperazines, produce noncarcinogenic nitrosamines. In addition, some compounds, e.g., 2,2,6,6-tetramethylpiperidine, are difficult to nitrosate due to steric hindrance in the molecule. Further examples of so-called 'safe' amines are given in Figure 1 (Eisenbrand *et al.*, 1986). It is worth mentioning that secondary amines with long aliphatic chains, e.g., dioctylamine, also have this property.

**Fig. 1. Selection of 'safe' amines: the corresponding nitrosamines were shown to be noncarcinogenic.**



Substitution of 'safe' amines for traditional accelerators, by replacing the common amino components (dimethylamine, morpholine and others), should result in compounds that retain the general properties of their substance class. Thus, their ability to act as vulcanization accelerators should not be impaired and the formation of carcinogenic nitroso compounds with ambient nitrogen oxides or other nitrosating agents would be avoided.

### Mutagenicity testing of the corresponding nitrosamines

Nine, in part newly synthesized, nitrosamines derived from 'safe' amines (see Table 1) were investigated for mutagenicity in *S. typhimurium* TA1535. The tests were performed essentially as described by Ames *et al.* (1975) in the presence of a metabolic activation system (9000  $\times$  *g* supernatant) with slight modifications (Maron & Ames, 1983). The nitrosamines were dissolved in spectrophotometric grade dimethyl sulfoxide in equimolar concentrations. NDMA (Aldrich), dissolved in twice-distilled water, was used as positive control. The preincubation test was carried out according to a slightly modified method of Yahagi *et al.* (1977).

A significant, reproducible increase in colony numbers was induced by NDMA (positive control), *N*-nitroso-*tert*-hexylbutylamine (NtHBA) and, to a lesser extent, by *N*-nitrosodioctylamine (NDOA). The lack of dose dependency and toxicity at higher doses of NDOA may be due to the observed precipitation of this compound. The activity of all the other nitrosamines was clearly weaker; at subtoxic doses, for example, *N*-nitroso-*tert*-butylmethylamine (NtBMA), *N*-nitroso-*tert*-amylmethylamine (NtAMA) and *N*-nitroso-*tert*-amylpropylamine (NtAPA) were shown to be nonmutagenic, and *N*-nitroso-2,2,6,6-tetramethylpiperidine (NTMP), which cannot be activated in the  $\alpha$ -position to nitrogen, was inactive. The results for the six nitrosamines that had biological effects (including NDMA) are shown in Figure 2.

These results are in agreement with those of studies by Druckrey *et al.* (1967), Rao *et al.* (1977) and Gold *et al.* (1981), who also demonstrated that alkyl-*tert*-butylnitrosamines are devoid of biological activity because of the nature of the tertiary butyl group. However, under specific experimental conditions, genotoxic effects may be observed due to liberated aldehydes (Pool *et al.*, 1984a). Further studies with *n*-alkyl-*tert*-amyl and *n*-alkyl-*tert*-hexyl nitrosamines are necessary to clarify the importance of the carbon chain length. NtHBA must undoubtedly be qualified as mutagenic.



**Table 1. Nitrosamines<sup>a</sup> investigated in *S. typhimurium* TA1535<sup>b</sup>**

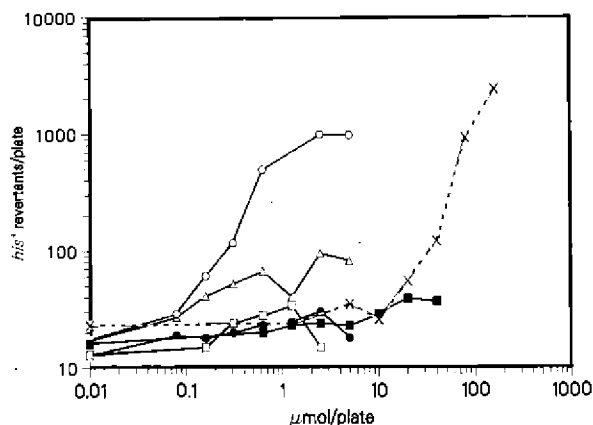
Compound	Abbreviation
<i>N</i> -Nitroso- <i>tert</i> -butylmethylamine	NtBMA
<i>N</i> -Nitroso- <i>tert</i> -butylethylamine	NtBEA
<i>N</i> -Nitroso- <i>tert</i> -butylbutylamine	NtBBA
<i>N</i> -Nitroso- <i>tert</i> -amylmethylamine <sup>c</sup>	NtAMA
<i>N</i> -Nitroso- <i>tert</i> -amylpropylamine <sup>c</sup>	NtAPA
<i>N</i> -Nitroso- <i>tert</i> -amylbutylamine <sup>c</sup>	NtABA
<i>N</i> -Nitroso- <i>tert</i> -hexylbutylamine <sup>c</sup>	NtHBA
<i>N</i> -Nitroso-2,2,6,6-tetramethylpiperidine	NTMP
<i>N</i> -Nitrosodioctylamine	NDOA

<sup>a</sup>Synthesis performed at the Institute of Toxicology and Chemotherapy, German Cancer Research Centre, Heidelberg

<sup>b</sup>Mutagenicity testing performed at the National Institute of Hygiene, Budapest, in collaboration with G. Török, A. Pintér and A. Surján

<sup>c</sup>Quaternary  $\alpha$ -carbon

molar amounts of amine, carbon disulfide and sodium hydroxide (Bögemann *et al.*, 1955). Because of their thermal instability, these products were mostly converted to the more stable zinc salts, which are normally used in industry. Pure dithiocarbamates were not obtained; slight contamination by inorganic salts, which could not be removed by recrystallization or modifications of the work-up procedure, was observed. Resolution of this problem is currently in progress.

**Fig. 2. Mutagenicity of nitrosamines derived from 'safe' amines; ■, NtBEA; ○, NtABA; △, NDOA; □, NtAPA; ◊, NtHBA; ×, NDMA**

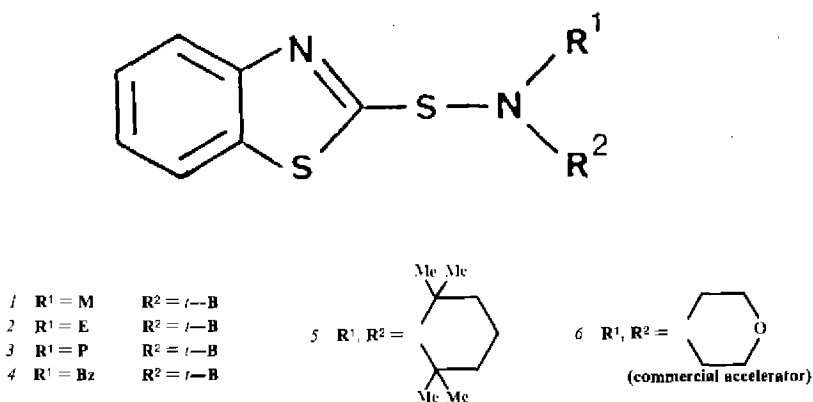
### Synthesis of new sulfenamide and dithiocarbamate accelerators

A series of about 20 sulfenamides and dithiocarbamates derived from 'safe' amines was synthesized (Fig. 3). The sulfenamides were prepared by oxidizing 2-mercapto-benzothiazole with sodium hypochlorite in the presence of a four- to five-fold excess of the relevant amine (Banks & Wiseman, 1968). The products were separated from the reaction mixture by column chromatography and crystallized from petroleum ether and diisopropylether, respectively; 50-70% yields of analytically pure substances were obtained. The sodium dithiocarbamates were synthesized by the reaction of equi-

### Toxicological studies

Ames' tests of the new sulfenamides and dithiocarbamates are still in progress. The nitrosation potential of sulfenamides 1-5 (Fig. 3) in acidic and neutral media has been investigated and compared to that of the morpholide, 6, which is frequently used as a commercial accelerator. The nitrosatability of these compounds was determined both at room and elevated temperature (100°C) to simulate vulcanization conditions. The experiments were performed as follows: 15 mg of the corresponding sulfenamide were dissolved in 1.5 ml of analytical-grade dioxane and added dropwise to 298.5 ml of an aqueous standard migration solution. (total

Fig. 3. Examples of new vulcanization accelerators (benzothiazole sulfenamides) based on 'safe' amines



volume, 300 ml), containing 0.5 g sodium chloride, 0.5 g potassium chloride and 20, 65 or 200 ppm nitrite. No precipitation of organic material was observed.

From each probe, smaller volumes (18 ml) were taken for the following procedures: (i) nitrosation at room temperature: the mixture was nitrosated for 30 min at pH 6-7 or pH 1, 2, 3 or 4. Nitrosation was terminated by addition of sodium hydroxide. Nitrosamines were extracted and determined according to the method of Spiegelhalter *et al.* (1983). (ii) Nitrosation at 100°C: the neutral solution was incubated at 100°C for 2.5 h. After cooling to room temperature, the pH was adjusted to 1, 2, 3 or 4. Nitrosation was stopped after 30 min by adding sodium hydroxide.

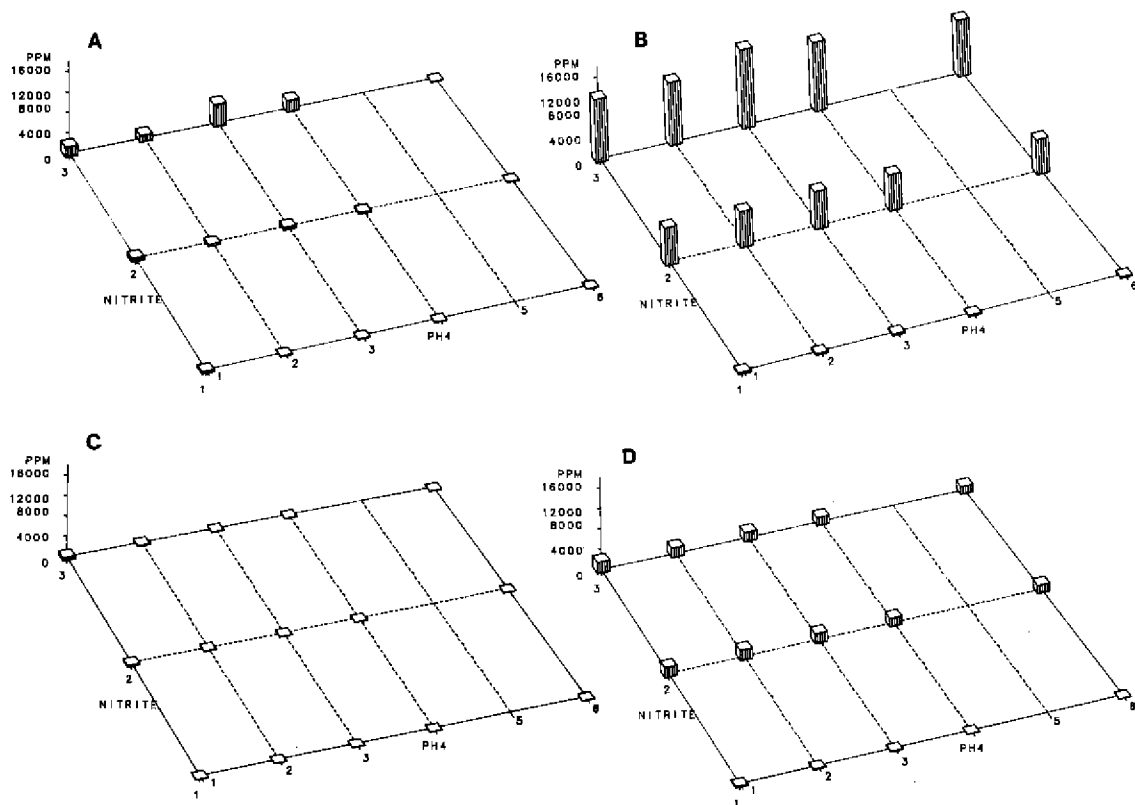
Figure 4 presents a comparison of the nitrosation potential of sulfenamide 1 and the commercially available accelerator 6. Only the corresponding nitrosamines, namely NtBMA (from 1) and NMOR (from 6) were detected. As expected, NMOR was found in four to seven times higher concentrations than NtBMA on average (at both temperatures). At room temperature, both nitrosation reactions show a typical pH-dependency, with maxima at pH 1 (1) or 3 (6), obviously due to the presence of free amines in the mixture. The latter may be formed by acidic hydrolysis of the amide substrates. No nitrosation occurred at pH 6-7.

At 100°C, the nitrosation characteristics changed completely. Appreciable amounts of N-nitrosamines — up to 11 000 ppm NMOR and 1500 ppm NtBMA — were formed even under neutral conditions. The increase in nitrosamine formation after cooling to room temperature and acidifying to pH 1, 2, 3 or 4 was comparably low.

This reflects the industrial situation, in which rubber mixtures, initially almost free of nitroso compounds, show high nitrosamine concentrations when brought into contact with nitrosating agents during the vulcanization process. The mechanism of this high-temperature reaction is still unknown but is apparently not the same as that observed under normal conditions. Perhaps, reactive intermediates, generated by partial decomposition of accelerator molecules, are responsible for this unusual type of nitrosation in the absence of an electrophilic agent.

The nitrosatability of the other sulfenamide derivatives, 2-5, was still lower than that observed for compound 1. Thus, these substances do not only form noncarcinogenic *N*-nitrosamines but are substantially less nitrosatable than the sulfenamide accelerators currently in use. The new compounds therefore seem suitable substitutes for the traditional vulcanization accelerators used in the rubber and tyre industry.

**Fig. 4.** Nitrite- and pH-dependencies of the nitrosation of 2-(morpholiniothio)benzothiazole, 6 (A: 25°C; B: 100°C) and 2-(2-*N*-methyl-1-*N*-*tert*-butyl)benzothiazole sulfenamide, 1 (C: 25°C; D: 100°C)



Nitrosamine concentrations are given in ppm = mg nitrosamine/kg accelerator; nitrite concentrations 1, 2 and 3 correspond to 20, 65 and 200 ppm  $\text{NO}_2^-$ .

# **N-NITROSAMINES AND NITROSATABLE COMPOUNDS IN RUBBER NIPPLES, PACIFIERS AND OTHER RUBBER AND PLASTIC COMMODITIES**

**G. Ellen & E.T. Sahertian**

*National Institute of Public Health and Environmental Hygiene,  
PO Box 1, 3720 BA Bilthoven, The Netherlands*

In 1983 and 1985, rubber teats and pacifiers from the Dutch market were analysed for *N*-nitrosamines (NA) and nitrosatable compounds (NC) by extraction with an artificial saliva test solution (24 h, 40°C). NC were determined as NA (gas chromatography-thermal energy analysis) after nitrosation. In 1983, the total content of NA and NC varied from 4-40 µg/kg and 50-3700 µg/kg, respectively (18 samples). In 1985, NA and NC varied from 3-94 µg/kg and 26-5100 µg/kg, respectively (20 samples). According to Dutch legislation, teats may contain no more than 1 µg/kg and 20 µg/kg NA and NC. Of other rubber and plastic commodities analysed, balloons contained levels of up to 120 µg/kg and 2800 µg/kg NA and NC.

Since the early 1980s, it has been known that rubber teats for baby bottles may contain NA and NC, which are extractable in aqueous solutions (Ireland *et al.*, 1980; Spiegelhalter & Preussmann, 1982). Because avoidable exposure of infants and young children to carcinogens is inadmissible, several countries have set limits for the extractable amounts of NA in rubber nipples and pacifiers. In the Netherlands, from 1983 on, migration from nipples into a saliva test solution may not exceed 1 µg/kg for NA and 20 µg/kg for NC (NC measured as NA after nitrosation). In 1983 and 1985, nipples and pacifiers from as many manufacturers as could be found were purchased in retail shops, to check whether they met the Dutch limits. The teats and pacifiers were analysed by the method of Spiegelhalter (1983), which involves extraction (24 h, 40°C) with an artificial saliva solution, nitrosation of a part of the extract to convert NC into NA, clean-up and measurement with gas chromatography-thermal energy analysis.

The results, presented as the sum of the contents of individual NA and NC (NC expressed as NA), are summarized in Table 1. The individual NA detected were *N*-nitrosodimethylamine, *N*-nitrosodiethylamine, *N*-nitrosodi-*n*-butylamine, *N*-nitrosopiperidine, *N*-nitrosomorpholine, *N*-nitrosophenylmethylamine and *N*-nitrosoethylphenylamine, of which *N*-nitrosodi-*n*-butylamine, *N*-nitrosophenylmethylamine and *N*-nitrosoethylphenylamine were found particularly frequently and at higher levels than the other NA. Table 1 shows that none of the specimens analysed in 1983 or in 1985 met the Dutch regulations. However, two samples of nipples of natural rubber, obtained recently directly from the manufacturer/importer, were found to contain amounts of extractable NA and NC no higher than those allowed by the Dutch regulation. This demonstrates that at least two manufacturers have taken successful measures to reduce levels of NA and NC.

**Table 1. *N*-Nitrosamines (NA) and nitrosatable compounds (NC) in nipples pacifiers of various brands, sampled in 1983 and 1985**

Brand (code)	Origin	Total amount of NA ( $\mu\text{g}/\text{kg}$ rubber)		Total amount of NC (as NA, $\mu\text{g}/\text{kg}$ rubber)	
		1983	1985	1983	1985
<i>Nipples</i>					
A	France	39	56	1100	2000
B	USA	22	20	330	440
C <sup>a</sup>	UK	19	10	150	99
D <sup>a</sup>	France	32	38	870	1200
E <sup>a</sup>	Switzerland	8	12	74	32
F	Netherlands	7	25	330	160
G <sup>a</sup>	Netherlands	6	13	50	78
H	Federal Republic of Germany	12	- <sup>c</sup>	150	-
I	Italy	21	3	2400	2800
J	France	32	-	440	-
K	Unknown	40	-	280	-
L	Italy	4	36	3400	4900
M <sup>a,b</sup>	Sweden	-	17	-	26
R	France	-	42	-	3600
S	UK	-	55	-	690
<i>Pacifiers</i>					
C	UK	12	-	270	-
E <sup>a</sup>	Switzerland	7	17	660	140
I	Italy	-	53	-	220
L <sup>a</sup>	Italy	-	15	-	42
M <sup>a</sup>	Sweden	10	44	3700	5100
N	Spain	18	-	1200	-
O <sup>a</sup>	Austria	10	46	68	320
S	UK	-	12	-	330
T	Unknown	-	94	-	740
U	Netherlands	-	29	-	4900
X	Unknown	11	-	420	-

<sup>a</sup>More than one type of nipple/pacifier of this brand was analysed in 1983 and/or 1985; results given are mean values for the various types.

<sup>b</sup>These nipples were made from silicone rubber; all the other nipples and pacifiers were made from natural (latex) rubber.

<sup>c</sup>-. No sample of this brand available in this year

Analyses of other rubber and plastic commodities, e.g., toys, pencil-erasers, kitchen utensils, balloons, gloves and medical appliances, such as catheters, showed that balloons contained high levels of extractable NA and NC, especially *N*-nitrosodiethylamine, at levels of up to 110 and 2800  $\mu\text{g}/\text{kg}$ , respectively. In plastic products, only trace amounts of NA and NC were detected occasionally.

#### Acknowledgements

This work was carried out on behalf of the Chief Officer of Public Health (Foodstuffs) under projects 648215 and 648503.

# POSSIBLE UNDERESTIMATION OF NITROSATABLE AMINE LEVELS IN ARTIFICIAL SALIVA EXTRACTS OF CHILDREN'S RUBBER PACIFIERS AND BABY-BOTTLE TEATS

M. Castegnaro<sup>1</sup>, J.R.A. Pollock<sup>2</sup> & M. Friesen<sup>1</sup>

<sup>1</sup>International Agency for Research on Cancer, 150 cours Albert Thomas, 69372 Lyon, France; and <sup>2</sup>Pollock & Pool Ltd, Ladbroke Close, Woodley, Reading RG5 4DX, UK

Children's pacifiers and baby-bottle nipples from various countries were analysed for their content of *N*-nitrosamines and nitrosatable amines. Using a method involving extraction with artificial saliva, several nitrosamines including *N*-nitrosodi-*n*-butylamine (NDBA), *N*-nitrosodiethylamine (NDEA), *N*-nitrosodimethylamine (NDMA) and *N*-nitrosomorpholine (NMOR) were detected in addition to the three nitrosatable amines dibutylamine (DBA), diethylamine (DEA) and dimethylamine (DMA). Upon nitrosation in artificial saliva, these amines produced not only the related *N*-nitrosamines but also relatively high levels of the corresponding nitramines — *N*-nitrodibutylamine (NTDBA), *N*-nitrodiethylamine (NTDEA) and *N*-nitrodimethylamine (NTDMA). Thus, both *N*-nitramines and *N*-nitrosamines should be measured after nitrosation; otherwise, the method probably underestimates the quantities of nitrosatable amines present in artificial saliva extracts. Whether *N*-nitramines, some of which have been shown to be both mutagenic and carcinogenic, are formed in the saliva of babies exposed to these products remains to be confirmed.

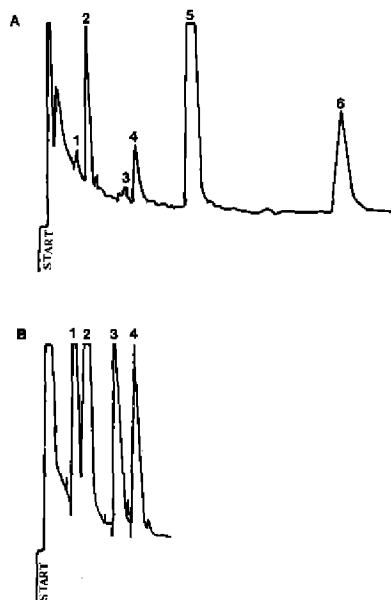
Since the first published reports, in the early 1980s of the presence of *N*-nitroso compounds in rubber baby teats and pacifiers (Ireland *et al.*, 1980; Spiegelhalter & Preussmann, 1982), a number of publications have appeared in the literature reporting the presence of NDMA, NDEA, NDBA, NPIP, NMOR, *N*-nitrosophenylmethylamine and *N*-nitrosoethylphenylamine. Basically, two types of methods have been proposed in the literature for the determination of these *N*-nitroso compounds. The first is based on the method of Havery and Fazio (1982) and involves extraction of nitrosamines from rubber products using an organic solvent (e.g., dichloromethane). Several amended versions have appeared (Sen *et al.*, 1984; Thompson *et al.*, 1984). In the second method (Spiegelhalter, 1983), only those *N*-nitrosamines that migrate from rubber into an artificial saliva extracting medium are estimated, but, in addition, this method measures nitrosatable substances that have migrated into the artificial saliva and can potentially be nitrosated *in vivo* to form *N*-nitrosamines.

## Experiments and results

In the migration test, the analysis of nitrosatable compounds is carried out by acidification of an artificial saliva extract of rubber products containing about 20 ppm nitrite and extraction of the medium (rendered alkaline) after 30 min by an excess of sodium

hydroxide solution. In a number of samples, peaks that did not correspond to the *N*-nitrosamines found before nitrosation were detected (Fig. 1A, peaks 3,4,5 and Fig. 1B, peaks 3,4). These peaks, responding to the thermal energy analyser, could not have derived from substances that had been employed in the formulations used to prepare the rubber. It was interesting to note that in no case were these compounds present before acidification of the artificial saliva extracts. The number of these new compounds, when they appeared, was often equivalent to the number of the original *N*-nitrosamines formed; the respective retention time of each compound when compared to that of the corresponding *N*-nitrosamine formed was about double. In 1980, we demonstrated (Walker & Castegnaro, 1980) that nitramines gave positive responses in the thermal energy analyser and had, under the gas chromatographic conditions used at that time, retention times 1.7 to 1.9 times greater than those of the corresponding *N*-nitrosamines.

**Fig. 1. Gas chromatographic profiles of nitrosatable compounds in two samples of rubber teats**



1, NDMA; 2, NDEA; 5, NDDBA

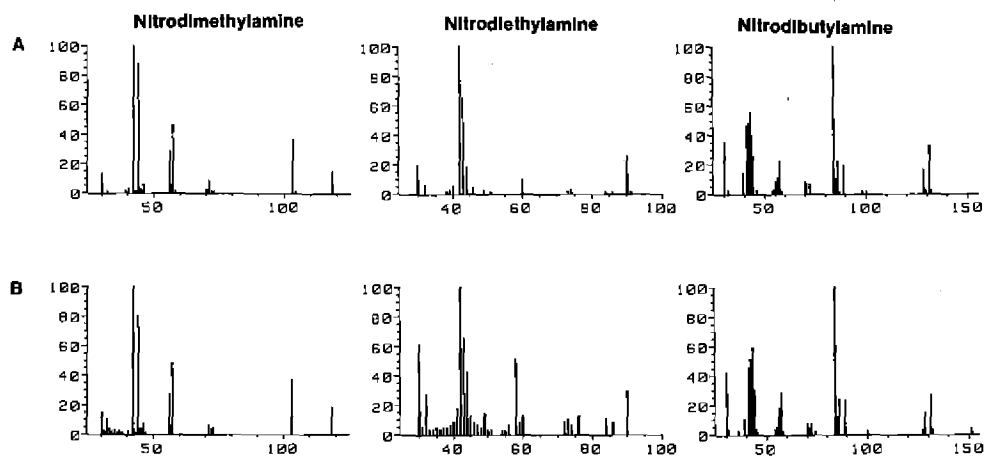
NTDMA, NTDEA and NTDBA were synthesized by oxidation of the corresponding *N*-nitrosamine with peroxotrifluoroacetic acid (Althorpe *et al.*, 1970), chromatographed under two sets of conditions with thermal energy analysis, and their retention time compared with those of the unknowns. In all cases the retention times of compounds 3, 4 and 6 (Fig. 1) corresponded to those of NTDMA, NTDEA and NTDBA.

The identity of the three unknown compounds was further confirmed by gas chromatography/mass spectrometry, using the following conditions: gas chromatography column: capillary OV101, 12 m long, 0.2 mm i.d., film thickness 0.3  $\mu$ m; column temperature: 30°C isothermal for 3 min then programmed at 20°C/min to 60°C then 5°C/min to 180°C; injector temperature, 200°C; interface temperature, 200°C; splitless injection; mass range, 20-600 amu. Gas chromatographic retention times and mass spectra of compounds 3, 4, 6 were identical to those of NTDMA, NTDEA and NTDBA (see Fig. 2).

### Conclusions and discussion

We have demonstrated that, in a number of samples, nitramines are produced during the analysis of nitrosatable compounds in artificial saliva. On a number of gas chromatographic columns, the behaviour of NTDMA is similar to that of *N*-nitrosodiisopropylamine, used as a standard in the method. This can lead to an incorrect assessment of the recovery, by excess, which, in turn, will provide an underestimation of nitrosatable compounds as *N*-nitrosamine. Moreover, the fraction of nitrosatable compounds converted to nitramines,

Fig. 2. Mass spectra of nitramine reference samples (A) and test material with corresponding gas chromatographic retention times (B)



if not taken into account, will also lead to underestimation of nitrosatables. Only on the basis of the latter consideration, the underestimation can be as much as 45%, depending on the sample formulation.

The reasons for the formation of these nitramines are not yet clear. Two hypotheses can be offered: (1) oxidation of the *N*-nitrosamines formed during the nitrosation step and (2) direct nitration of the amines or other substances extracted from the rubber.

Under the conditions of analysis, the presence of *tert*-butylhydroperoxide did not cause formation of nitramines during nitrosation of the corresponding amine. An aqueous saliva extract that produced 546  $\mu\text{g}/\text{kg}$  NDMA and 310  $\mu\text{g}/\text{kg}$  NTDMA (concentrations refer to weight of rubber) after acidification was then spiked with 750  $\mu\text{g}/\text{kg}$  NDEA and treated by the normal procedure (acidification, 30-min reaction, addition of excess sodium hydroxide); no NTDEA was found.

These two results show that it is most likely that the formation of nitramines during the analysis of nitrosatable amines does not proceed through a nitrosation step but rather through a direct nitration pathway. Since nitramine formation depends on the type of rubber, one may envisage oxidation of the nitrosating species to nitrogen tetroxide, which will then play the role of a direct nitrating agent.



**ESCHERICHIA COLI INFECTION OF THE  
URINARY BLADDER: INDUCTION OF TUMOURS IN RATS  
RECEIVING NITROSAMINE PRECURSORS AND  
AUGMENTATION OF BLADDER CARCINOGENESIS BY  
N-NITROSOBUTYL (4-HYDROXYBUTYL)AMINE**

N.A. Higgy<sup>1,4</sup>, A.K. Verma<sup>1</sup>, E. Ertürk<sup>1</sup>, T.D. Oberley<sup>2</sup>,  
A.A. El-Aaser<sup>3</sup>, M.M. El-Merzabani<sup>3</sup> & G.T. Bryan<sup>1</sup>

<sup>1</sup>Department of Human Oncology, University of Wisconsin  
Clinical Cancer Center, Madison, Wisconsin 53792, USA;

<sup>2</sup>Department of Pathology, University of Wisconsin Medical School,  
Madison, Wisconsin 53792, USA; and <sup>3</sup>Cancer Biology Department,  
National Cancer Institute, Cairo University, Cairo, Egypt

Experimental introduction of *Escherichia coli* type 04 into the subserosa of the urinary bladder of female Fischer 344 rats produced chronic bacterial infection in more than 90% of animals. Groups of rats with bacterial infection were given sodium nitrate and either piperazine (Group 1) or dibutylamine (Group 2) in the drinking-water. Control, noninfected animals received nitrate and either piperazine (Group 3) or dibutylamine (Group 4). At 40 weeks, transitional-cell carcinomas of the bladder were detected in 9/30 rats in Group 1 compared to 0/34 in Group 3 ( $p < 0.0005$ ), and in 11/34 rats in Group 2 compared to 0/32 in Group 4 ( $p < 0.0003$ ). Early changes were examined by scanning and transmission electron microscopy as well as autoradiography. Preneoplastic liver foci were detected in infected groups of animals receiving amine and nitrate, indicating reabsorption of the carcinogen synthesized *in situ* to induce distant organ transformation. In another experiment, *E. coli* infection augmented bladder carcinogenesis by *N*-nitrosobutyl(4-hydroxybutyl)amine (NBHBA), as indicated by earlier appearance of bladder tumours (six weeks compared to nine weeks) and, after 25 weeks, higher incidences of transitional-cell carcinomas (41/46 compared to 39/53,  $p < 0.05$ ), squamous metaplasia (43% compared to 9%,  $p < 0.0001$ ), glandular metaplasia (26% compared to 13%,  $p < 0.05$ ) and muscle invasion (30% compared to 11%,  $p < 0.01$ ) in the *E. coli*-infected group receiving carcinogen compared to the noninfected group receiving carcinogen, respectively. These results indicate that bacterial infection of the urinary bladder may play a major role in bladder carcinogenesis, both by helping *in-situ* nitrosamine synthesis and by augmenting carcinogenesis by nitrosamines.

Bacterial infection has been reported to increase the risk of developing bladder cancer in individuals with a history of chronic or repeated cystitis (Radomski *et al.*, 1978; Kantor *et al.*, 1984), in paraplegics (Melzak, 1966) and in patients infested with the parasite *Schistosoma haematobium*, who also have a coincident chronic bacterial infection (Elsebai, 1978; El-Aaser *et al.*, 1979). These studies emphasize that bacterial infection may indeed be a major factor in bladder carcinogenesis.

<sup>4</sup>Present address: Cancer Biology Department, National Cancer Institute, Cairo University, Cairo, Egypt

We have examined the effects of long-term administration of secondary amines and nitrate to rats with experimentally produced *Escherichia coli* infection in the urinary bladder, to investigate whether the process of carcinogenesis initiated in the urinary bladder by low doses of a urine-borne carcinogen, *N*-nitrosobutyl(4-hydroxybutyl)amine (NBHBA), is accelerated by the proliferative changes in the urothelium that develop in response to bacterial infection.

Inbred female Fischer 344 rats, seven to eight weeks old were divided into six groups of 50 rats each. Rats in group 1 were controls, while rats in groups 2, 3 and 4 were infected with *E. coli* for week one of the experiment. They were anaesthetized with intraperitoneally injected Nembutal (10 µg/g body weight), then the skin and peritoneum were incised to expose the urinary bladder, and an *E. coli* (type 04) suspension ( $2 \times 10^6$  bacteria) in 0.2 ml saline was injected subserosally into the wall of the exposed bladders. Designated groups of rats were given 500 ppm nitrate and 125 ppm piperazine or dibutylamine in their drinking-water for 40 weeks. In a second study, rats were divided into four groups. Rats in group 1 were untreated controls, rats in groups 2 and 4 were infected with *E. coli* as described above, and those in groups 3 and 4 were given 0.025% NBHBA solution in drinking-water containing 0.05% Tween 80 for ten weeks from brown bottles, which were filled twice weekly with freshly prepared solution.

The urinary bladders were examined by light, scanning and transmission electron microscopy, as well as by autoradiography.

#### **Induction of bladder tumours in infected rats receiving amines and nitrate**

The lesions observed in the urinary bladders of rats in the first study, killed 40 weeks after start of infection, are shown in Table 1. In groups 3 and 4, which received piperazine or dibutylamine and nitrate after bacterial infection, urinary bladder hyperplasia progressed to carcinoma *in situ* by week 25 after infection with *E. coli*. After 40 weeks, transitional-cell carcinomas were detected in bladders of rats in groups 3 and 4, with muscle invasion and squamous metaplasia and keratinization. None of these changes was seen in any of the other groups. Scanning electron microscopy and transmission electron microscopy provided complementary evidence of a malignant urothelium.

The carcinogen that had apparently been formed in the urine from its precursors and caused the bladder changes appeared to have been absorbed into the circulation to induce preneoplastic foci in the liver, particularly in rats of group 4, where cells were irregular in pattern, size and shape. No preneoplastic or neoplastic change was detected in any other organ examined.

This is the first report of transitional-cell carcinoma resulting after experimental infection of rats with *E. coli* in the urinary bladder and administration of nitrate and either piperazine or dibutylamine for 40 weeks. Nitrosamines have been detected in the urine of rats with experimental bladder infection that were fed piperidine and nitrate (Hawksworth & Hill, 1974). Piperazine, a drug given commonly to children to eliminate pinworms, can undergo nitrosation to form mononitrosopiperazine and dinitrosopiperazine, both of which are carcinogenic to laboratory animals (Druckrey *et al.*, 1967; Garcia *et al.*, 1970). The concurrent administration of piperazine and nitrate has been reported to induce a significant number of lung adenomas in Swiss mice (Greenblatt *et al.*, 1971). In our study, no change was detected in the lung but preneoplastic liver foci were found, indicating the possible absorption of the nitrosamines formed *in situ* from the bladder to the general circulation.

**Table 1. Bladder and liver lesions in rats 40 weeks after *Escherichia coli* infection and administration of nitrate and amine<sup>a</sup>**

Group	Treatment	Effective no of rats <sup>b</sup>	Bladder				Preneoplastic liver foci
			Stones	Simple hyperplasia	Nodular or papillary hyperplasia	Transitional-cell carcinoma	
1	Untreated controls	35	0	0	0	0	0
2	<i>E. coli</i> -infected	30	0	5	6	0	0
3	<i>E. coli</i> + nitrate + piperazine	30	2	0	21 <sup>c</sup>	9 <sup>d</sup>	7 <sup>e</sup>
4	<i>E. coli</i> + nitrate + dibutylamine	34	3	0	23 <sup>f</sup>	11 <sup>g</sup>	12 <sup>h</sup>
5	Nitrate + piperazine	34	0	8	0 <sup>c</sup>	0 <sup>d</sup>	2 <sup>e</sup>
6	Nitrate + dibutylamine	32	0	6	0 <sup>f</sup>	0 <sup>g</sup>	2 <sup>h</sup>

<sup>a</sup>Numbers of rats are given under the column of most advanced lesion.

<sup>b</sup>Number of rats killed at 40 weeks

<sup>c</sup> $p < 0.002$

<sup>d</sup> $p < 0.01$

<sup>e</sup> $p < 0.0002$

<sup>f</sup> $p < 0.04$

<sup>g</sup> $p < 0.004$

<sup>h</sup> $p < 0.0005$

### Augmentation of carcinogenesis by NBHBA

In the second study, the lesions observed in urinary bladders of rats in group 4 were more advanced than those in group 3. Bladder tumours appeared earlier, were more frequent and exhibited squamous and glandular metaplasia and muscle invasion more significantly in rats in group 4 than in those in group 3.

In rats infected with *E. coli* (group 3), a 15-fold increase in DNA synthesis occurred 48 h after infection, then decreased to slightly higher than basal levels by two weeks. DNA synthesis then started to increase again gradually, and continued to do so until the end of the experiment. In group 4, a marked increase (20-fold) was seen at 48 h, which did not decline but continued to rise steadily. Two-way analysis of variance showed significant nonadditivity ( $p < 0.005$ ), indicating that NBHBA and bacteria have a synergistic effect in increasing

DNA synthesis. These increments coincided with the marked hyperplasia taking place in the urothelium, as indicated by increased labelling by <sup>3</sup>H-thymidine of tissues examined by autoradiography.

Infection by *E. coli* augmented tumour induction by the bladder carcinogen NBHBA and shortened the tumour latent period. The time required for tumour development by NBHBA was reduced in the presence of the bacterial infection. Chronic bacterial infection provides a continuous regenerative stimulus. In addition to increasing the susceptibility of the urothelium to initiation, this proliferative stimulus may act as a promoting factor for previously initiated urothelial cells.

**Acknowledgement**

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## N-NITROSAMINE FORMATION IN URINARY-TRACT INFECTIONS

H. Ohshima<sup>1</sup>, S. Calmels<sup>1</sup>, B. Pignatelli<sup>1</sup>, P. Vincent<sup>2</sup> & H. Bartsch<sup>1</sup>

<sup>1</sup>International Agency for Research on Cancer, 150 cours Albert Thomas, Lyon;  
and <sup>2</sup>Hôpital de la Croix Rousse, Lyon, France

Urine samples from 31 patients with urinary-tract infections and from 31 controls were analysed for volatile nitrosamines, *N*-nitrosamino acids, total *N*-nitroso compounds as a group, and nitrite/nitrate. The concentration of *N*-nitrosodimethylamine was significantly elevated in urines infected with *Escherichia coli*, *Proteus mirabilis* and *Klebsiella pneumoniae*. The levels of nitrite, *N*-nitrosoproline and total *N*-nitroso compounds, when expressed as the amount per mol creatinine, were also significantly increased in patients with bacteriuria. Several bacterial strains were capable of catalysing nitrosation of morpholine at neutral pH. These results suggest that *N*-nitroso compounds can be formed *in vivo* in the infected bladder, which could explain the association between urinary-tract infections and increased risk for bladder cancer.

Several epidemiological studies have suggested that infections of the urinary tract may be a risk factor for cancer of the bladder (Wynder *et al.*, 1963; Dunham *et al.*, 1968; Howe *et al.*, 1980; Kantor *et al.*, 1984). Nitrosamine formation by bacteria in the bladder has been hypothesized to be an associated risk factor (Hawksworth & Hill, 1974; Hicks *et al.*, 1977; Radomski *et al.*, 1978; El-Merzabani *et al.*, 1979; Hicks *et al.*, 1982). However, no extensive study has been presented concerning the occurrence and formation of *N*-nitroso compounds in relation to the type and count of bacterial flora present in infected urine. In the present study, urine samples obtained from patients with urinary-tract infections and from healthy controls were analysed for volatile nitrosamines, nonvolatile nitrosamino acids, total *N*-nitroso compounds and nitrite/nitrate. The results were correlated with the bacterial count and the type of microorganism present in the urine.

### Urine samples and analysis

Samples of morning urine were collected from 31 patients with urinary-tract infections and from 31 control subjects with sterile urine. Characteristics (age, sex, smoking habits) of these subjects are given in Table 1, with the bacterial species and counts determined in their urines. Of 31 samples from patients with bacteriuria, 17 were infected with *Escherichia coli*, three with *Klebsiella pneumoniae*, and each of two samples with either *Staphylococcus coagulase*, *Proteus morgani*, *Pseudomonas aeruginosa* or *Candida tropicalis*. The remaining three samples were infected with *Klebsiella oxytoca*, *Proteus mirabilis* or *Streptococcus* spp.

The urine samples were analysed for nitrite/nitrate (Sen & Donaldson, 1978; Green *et al.*, 1982), creatinine (Varleg, 1967), volatile nitrosamines and nitrosamino acids, as described in the legend to Table 3. Some urine samples were also analysed for total

**Table 1. Characteristics of study subjects and bacteria identified in urine<sup>a</sup>**

Sample	No. of samples	Sex		Age (years)	Smoking habit (No. of subjects)		Bacterial count/ml (No. of samples)					
		Male	Female		Smoker	Non-smoker	>10 <sup>7</sup>	10 <sup>6</sup>	10 <sup>5</sup>	10 <sup>4</sup>	0	
<b>Bacteriuria</b>												
<i>E. coli</i>	17	1	16	53 (25-88)	2	15	11	3	2	1	0	0
<i>K. pneumoniae</i>	3	0	3	3, 81, 85	1	2	1	1	1	0	0	0
<i>S. coagulase</i>	2	1	1	18, 61	1	1	1	1	0	0	0	0
<i>P.morganii</i>	2	0	2	85, 86	2	2	1	0	0	1	0	0
<i>P. aeruginosa</i>	2	1	1	72, 80	0	2	1	1	0	0	0	0
<i>C. tropicalis</i>	2	2	0	59, 59	0	2	2	0	0	0	0	0
<i>K. oxytoca</i>	1	1	0	58	1	0	1	0	0	0	0	0
<i>P. mirabilis</i>	1	0	1	33	0	1	1	0	0	0	0	0
<i>Streptococcus</i> spp	1	0	1	17	0	1	1	0	0	0	0	0
Total bacteriuria	31	6	25	55 (3-88)	5	26	20	6	3	2	0	0
Sterile urine	31	8	23	47 (8-91)	5	26	0	0	0	0	0	31

<sup>a</sup>Mean (range) or individual values

*N*-nitroso compounds as a group according to the method of Walters *et al.* (1978, 1983), using a Thermal Energy Analyzer as detector (Castegnaro *et al.*, 1986). All results are expressed as concentrations (nmol or  $\mu$ mol of the compound per litre urine) and as the values corrected for creatinine concentrations ( $\mu$ mol or mmol per mol creatinine). Statistical analysis was carried out using the Wilcoxon test (Hollander & Wolfe, 1973).

### Nitrate and nitrite

Table 2 shows pH and levels of nitrite and nitrate in the urine. Nitrate levels (mean  $\pm$  SD, mmol/l) detected in sterile urines were  $1.08 \pm 0.74$  and were not significantly different from those for patients with bacteriuria ( $1.03 \pm 1.58$ ). Nitrite was detected in only one sample out of 31 sterile urines, whereas 15 samples out of 31 from patients with bacteriuria contained nitrite levels ranging from 10 to 220  $\mu$ mol/l ( $p < 0.001$ , Table 2).

### Volatile nitrosamines

The levels of volatile nitrosamines detected in samples from patients with bacteriuria and sterile urines are shown in Table 3. *N*-Nitrosodimethylamine (NDMA) was detected in 17 samples from patients with bacteriuria and 11 sterile urines; although the levels were not significantly different between the two groups, the urines infected with *E. coli* contained significantly higher levels of NDMA than sterile urines ( $p < 0.05$ ). Relatively large quantities of NDMA were found in three patients infected with *K. pneumoniae* and one patient with *P. mirabilis*. Samples infected with *S. coagulase*, *P.morganii*, *P. aeruginosa*, *K. oxytoca*, *Streptococcus* spp and *C. tropicalis* contained only trace or nondetectable levels of NDMA. *N*-Nitrosopyrrolidine was detected in four samples from patients with bacteriuria and two sterile urines, while *N*-nitrosopiperidine was present in ten samples from patients with bacteriuria and seven sterile urines (differences not significant). Thus, concentrations of the sum of the three volatile nitrosamines detected in samples from patients with bacteriuria were significantly higher than those for sterile urines ( $p < 0.05$ ).

Table 2. pH and levels of nitrite and nitrate in bacteriuria and sterile urines<sup>a,b</sup>

Sample	pH	Nitrite		Nitrate		No. of positive samples
		$\mu\text{mol/l}$	mmol/mol CRN	mmol/l	mmol/mol CRN	
Bacteriuria						
<i>E. coli</i>	6.17±1.01 (5.00-8.76)	76±96 (ND-330)**	10 (ND-54)***	1.28±2.15 (0.05-7.61)	139±96 (17-307)	17
<i>K. pneumoniae</i>	6.56, 6.09, 6.03	34, 20, ND	10, 8, ND	1.88, 0.32, 0.07	783, 75, 30	3
<i>S. coagulase</i>	5.53, 9.25	60, ND	10, ND	3.81, 0.19	431, 33	2
<i>P.morganii</i>	6.46, 7.37	ND, ND	ND, ND	0.29, 0.25	126, 71	2
<i>P. seruginosa</i>	5.17, 6.69	ND, ND	ND, ND	0.90, 0.19	143, 110	2
<i>C. tropicalis</i>	4.92, 5.14	ND	ND	0.53, 0.43	434, 181	2
<i>K. oxytoca</i>	8.58	ND	ND	0.45	79	1
<i>P. mirabilis</i>	6.92	100	7	1.00	70	1
<i>Streptococcus</i> spp	7.63	ND	ND	0.84	250	1
Total bacteriuria	6.36±1.14 (5.14-9.25)	47±79 (ND-330)***	10±11 (ND-54)**	1.03±1.58 (0.05-7.61)	174±171 (17-783)	31
Sterile urine	6.11±1.03 (4.78-8.28)	1±5 (ND-30)	0.4±2 (ND-12)	1.08±0.74 (0.29-2.80)	150±114 (19-540)	31

<sup>a</sup> Mean (range) or individual values; ND, not detected; CRN, creatinine<sup>b</sup> Comparison with the levels detected in sterile urines; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$

Table 3. Levels of volatile nitrosamines in bacteriuria and sterile urines<sup>a, b</sup>

Sample	NDMA		NPYR		NPIP		Sum	
	nmol/l	μmol/mol CRN	nmol/l	μmol/mol CRN	nmol/l	μmol/mol CRN	nmol/l	μmol/mol CRN
Bacteriuria								
<i>E. coli</i>	16.4±41.4 (ND-160)*	1.07±1.96 [12]	0.6±1.6 (ND-6.2)	0.18±0.61 [3] (ND-2.50)	2.2±3.9 (ND-14.7)	0.28±0.50 [7] (ND-1.49)	18.3±41.2 (ND-166)*	1.54±2.40 [14] (ND-8.37)
<i>K. pneumoniae</i>	75.4, 20.0, 1.2	17.8, 8.34, 0.54	4.2 ND, ND	1.80, ND, ND	ND, ND, ND	ND, ND, ND	75.4, 24.2, 1.2	17.8, 10.1, 0.54
<i>S. coagulase</i>	0.95, ND	0.18, ND	ND, ND	ND, ND	2.8, ND	0.49, ND	3.8, ND	0.67, ND
<i>P. Morganii</i>	ND, ND	ND, ND	ND, ND	ND, ND	ND, ND	ND, ND	ND, ND	ND, ND
<i>P. seruginosa</i>	ND, ND	ND, ND	ND, ND	ND, ND	3.3, ND	0.63, ND	3.3, ND	0.63, ND
<i>C. tropicalis</i>	ND, ND	ND, ND	ND, ND	ND, ND	ND, ND	ND, ND	ND, ND	ND, ND
<i>K. oxytoca</i>	ND	ND	ND	ND	ND	ND	ND	ND
<i>P. mirabilis</i>	31.4	2.16	ND	ND	ND	ND	31.4	2.16
<i>Streptococcus</i> spp	ND	ND	ND	ND	2.5	0.74	2.5	0.74
Total bacteriuria	12.7±32.7 (ND-160)	1.63±3.77 [17] (ND-17.8)	0.5±1.4 (ND-6.2)	0.16±0.55 [4] (ND-2.50)	1.5±3.1 (ND-14.7)	0.22±0.41 [10] (ND-1.49)	14.6±33.4 (ND-166)*	1.90±3.86 [21] (ND-17.8)
Sterile urine	1.8±3.7 (ND-18.9)	0.35±0.71 [11] (ND-3.38)	0.3±1.2 (ND-5.5)	0.08±0.30 [3] (ND-1.40)	0.6±1.4 (ND-5.79)	0.14±0.34 [7] (ND-1.67)	2.6±4.7 (ND-24.4)	0.56±0.99 [14] (ND-4.82)

<sup>a</sup> Mean ± SD (range) or individual values; [ ], no. of positive samples; ND, not detected; CRN, creatinine

<sup>b</sup> Comparison with the levels detected in sterile urines; \*,  $p < 0.05$

*N*-Nitrosamines were analysed as follows: two internal standards (*N*-nitrosopropylmethylamine and *N*-nitrosopropylamine) were added to 15-ml urine samples that had been treated with excess sulfuric acid. Sample were adjusted to pH 8-10 with 6 N sodium hydroxide solution and extracted three times with 30 ml dichloromethane. The combined dichloromethane extracts were dried over anhydrous sodium sulfate and evaporated in a Kuderna Danish evaporator at 50°C to analyse volatile nitrosamines. The aqueous phase remaining after extraction of volatile nitrosamines was acidified with 3.6 N sulfuric acid, then extracted three times with 40 ml dichloromethane:methanol (9:1, v/v) mixture for the analysis of nitrosamino acids. These *N*-nitrosamines were analysed by gas chromatography-thermal energy analysis, as reported previously (Calmeils *et al.*, 1985).



### *N*-Nitrosamino acids

The levels of *N*-nitrosamino acids detected in the urines are shown in Table 4. NPRO was detected in all samples (except for one sterile urine), in concentrations of 1.3-123.8 nmol/l in samples from patients with bacteriuria, with a mean  $\pm$  SD of  $15.6 \pm 25.3$  nmol/l; and nondetectable to 23.3 nmol/l, with a mean  $\pm$  SD of  $6.6 \pm 5.6$  nmol/l in sterile urines. Although the NPRO concentrations were not significantly different between the two groups, the levels of NPRO corrected for creatinine ( $\mu\text{mol/mol}$  creatinine) were higher in samples from patients with bacteriuria than in control urines ( $p < 0.05$ ). Patients infected with *E. coli* excreted greater amounts of NPRO in the urine than those without urinary-tract infections ( $p < 0.05$ ). Two sulfur-containing *N*-nitrosamino acids were also detected in some samples from patients with bacteriuria and sterile urines, however, their levels were not statistically different in the two groups.

### Total *N*-nitroso compounds

The method developed by Walters *et al.* (1978, 1983) was applied to determine total *N*-nitroso compounds as a group in the urine samples. Total *N*-nitroso compounds were extracted with ethyl acetate from urine treated with sulfamic acid and selectively denitrosated with hydrogen bromide to nitric oxide, which was determined using a Thermal Energy Analyzer (Walters *et al.*, 1978, 1983; Castegnaro *et al.*, 1986). Six control sterile urines and 13 from patients with bacteriuria (five *E. coli*, two *K. pneumoniae*, one *K. oxytoca*, one *P. mirabilis*, two *Streptococcus* spp, and two *C. tropicalis*) were examined. The total *N*-nitroso compound concentrations ranged from 0.03-10.81 (mean  $\pm$  SD,  $1.69 \pm 2.86$ )  $\mu\text{mol/l}$  for samples from patients with bacteriuria and from 0.01-2.93 (mean  $\pm$  SD,  $0.71 \pm 1.13$ )  $\mu\text{mol/l}$  for sterile urines. Although these concentrations were not significantly different, the amount of *N*-nitroso compounds corrected for creatinine ( $\text{mmol/mol}$  creatinine) were higher in samples from patients with bacteriuria than in sterile urines ( $p < 0.05$ ). It is noteworthy that the total *N*-nitroso compound concentrations are 3-3000 times greater than those for the sum of all volatile nitrosamines and nitrosamino acids detected in the same urine samples, which may suggest the occurrence of hitherto unknown *N*-nitroso compounds in the urine. However, it was shown recently that the procedure of Walters *et al.* (1978, 1983) may give false-positive responses which can arise from nitrite and nitrate present in the urine and may underestimate the amounts due to the occurrence of less easily extractable nitroso compounds in ethyl acetate (Pignatelli *et al.*, this volume). An improved method for the analysis of total *N*-nitroso compounds in urine is being developed similar to that for the analysis of gastric juice samples (Pignatelli *et al.*, this volume).

### Nitrosation *in vitro* by bacteria isolated from urinary-tract infections

Fourteen strains isolated from samples from patients with bacteriuria (eight *E. coli*, three *K. pneumoniae*, one *K. oxytoca*, one *P. aeruginosa* and one *P.morganii*) were examined for nitrosation of morpholine at pH 7.2. Except for two (one *E. coli* and one *K. pneumoniae*), all strains catalysed nitrosation, with specific activities ranging from 5-86 nmol NMOR formed per mg protein per hour (Calmels *et al.*, this volume). No apparent correlation, however, was observed between the nitrosation activity of the isolated bacteria and the levels of volatile nitrosamines, nitrosamino acids or total *N*-nitroso compounds detected in the urine samples from which bacteria strains were isolated. In addition, since the number of urine specimens infected with a specific type of bacterial species is limited, the

Table 4. Levels of *N*-nitrosamino acids detected in bacteriuria and sterile urine<sup>a, b</sup>

Sample	<i>N</i> -Nitrosoproline		<i>N</i> -Nitroso(2-methylthiazolidine) 4-carboxylic acid		<i>N</i> -Nitrosothiazolidine 4-carboxylic acid		Sum
	nmol/l	μmol/mol CRN	nmol/l	μmol/mol CRN	nmol/l	μmol/mol CRN	
<b>Bacteriuria</b>							
<i>E. coli</i>	20.6±32.0* (1.3-123.8)	2.23±2.70* [17] (0.28-11.46)	14.9±58.7 (ND-242.5)	2.01±7.30 [5] (ND-30.16)	77.8±163.0 (ND-486.2)	6.29±12.66 [13] (ND-40.93)	113.3±216.4 (1.3-567.2)
<i>K. pneumoniae</i>	8.5, 6.3, 1.5	15.97, 1.46, 0.76	140, ND, ND	3.32, ND, ND	119.2, 8.4, 3.8	28.08, 3.49, 1.92	139.4, 46.6, 5.4
<i>S. coagulans</i>	18.2, 3.3	2.08, 0.56	2.3, ND	0.27, 0	8.4, 0.8	0.93, 0.12	28.9, 4.1
<i>P. mirabilis</i>	5.2, 4.8	2.08, 1.32	ND, ND	ND, ND	5.8, 3.5	1.63, 1.51	10.5, 8.3
<i>P. aeruginosa</i>	5.2, 4.1	1.32, 0.49	ND, ND	ND, ND	14.3, ND	2.73, ND	19.6, 4.1
<i>C. tropicalis</i>	2.9, 2.1	1.74, 1.25	ND, ND	ND, ND	ND, ND	ND, ND	2.9, 2.1
<i>K. oxytoca</i>	34.0	5.35	17.3	2.77	70.6	11.10	122.0
<i>P. mirabilis</i>	4.1	0.28	ND	ND	25.2	1.74	29.3
<i>Streptococcus</i> spp	3.8	1.11	ND	ND	ND	ND	3.8
Total bacteriuria	15.6±25.3 (1.3-123.8)	2.37±3.33* [31] (0.28-15.97)	9.3±43.5 (ND-242.5)	1.31±5.44 [8] (ND-30.16)	51.0±124.8 (ND-486.2)	5.17±10.57 [23] (ND-40.93)	75.9±166.1 (1.3-567.2)
Sterile urine	6.6±5.6 (ND-23.3)	1.09±0.97 [30] (ND-3.82)	1.4±4.2 (ND-21.4)	0.29±1.08 [6] (ND-5.98)	11.8±19.4 (ND-74.1)	2.49±4.76 [21] (0.17-21.51)	19.8±24.9 (ND-88.2)

<sup>a</sup>Mean ± SD (range) or individual values are given; [ ], no. of positive samples; ND, not detected; CRN, creatinine

<sup>b</sup>Comparison with the levels detected in sterile urines; \*, *p* < 0.05

relationship between the bacterial count of a particular strain and the levels of *N*-nitroso compounds could not be evaluated in the present study. However, there was a marked tendency for the urines infected with high counts of *E. coli* to contain higher concentrations of NDMA and NPRO.

In conclusion, we have demonstrated that the concentrations of not only volatile nitrosamines such as NDMA but also of nonvolatile nitrosamino acids and total *N*-nitroso compounds are elevated in the urine of patients with urinary-tract infections, thus confirming the previous findings of Radomski *et al.* (1978), Hicks *et al.* (1977, 1982), El-Aaser *et al.* (1980) and Abdel-Tawab *et al.* (1986). It was also shown that various bacterial strains isolated from urinary-tract infections are capable of catalysing nitrosation of secondary amines *in vitro* (Calmels *et al.*, 1985, and this volume). These results suggest that *N*-nitroso compounds can be formed *in vivo* in the bladder of patients with urinary-tract infections, to increase the body burden of carcinogens through subsequent absorption of the compounds from the urinary bladder into the blood stream, as shown in rats by Hawksworth and Hill (1974). Recent epidemiological studies have shown a positive association between urinary-tract infections and bladder cancer, particularly squamous-cell carcinoma (Kantor *et al.*, 1984), and with an increased frequency of all cancer (Nordenstam *et al.*, 1986). In this respect, further studies are warranted to determine what types of *N*-nitroso compounds can be generated in relation to the type and count of bacterial species and to investigate the toxic and other adverse biological effects of these nitroso compounds.

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# **N-NITROSAMINE FORMATION BY MICROORGANISMS ISOLATED FROM HUMAN GASTRIC JUICE AND URINE: BIOCHEMICAL STUDIES ON BACTERIA-CATALYSED NITROSATION**

**S. Calmels<sup>1</sup>, H. Ohshima<sup>1</sup>, M. Crespi<sup>2</sup>, H. Leclerc<sup>3</sup>,  
C. Cattoen<sup>3</sup> & H. Bartsch<sup>1</sup>**

<sup>1</sup>*International Agency for Research on Cancer, 150 cours Albert Thomas, Lyon;*

<sup>2</sup>*The Regina Elena Institute for the Study and Therapy of Tumours  
Rome, Italy; and* <sup>3</sup>*INSERM, Villeneuve d'Ascq, France*

**Twelve out of 14 bacterial strains isolated from patients with urinary infections and nine out of 30 microorganisms isolated from gastric juice from patients with gastric achlorhydria were shown to catalyse the formation of *N*-nitrosomorpholine (NMOR) from nitrite and morpholine at neutral pH. The effects of various metal ions and cofactors on the bacterial nitrosation reaction was investigated. The presence of nitrate in the culture medium was required to induce nitrosating activity in bacteria, but low nitrate concentrations inhibited the nitrosation reaction.**

Although bacteria-catalysed nitrosation has now been clearly demonstrated (Suzuki & Mitsuoka, 1984; Calmels *et al.*, 1985; Leach *et al.*, 1985a), the biochemical properties of the presumed enzyme involved and the clinical relevance of this reaction in humans have not been studied in detail. For these reasons, we have been continuing to screen for nitrosating bacteria in urine from human subjects suffering from urinary infections and from patients with gastric achlorhydria. In addition, we have studied the effects of culture conditions, of various cofactors and of metal ions on the bacterial nitrosation reaction.

## **Nitrosation-proficient bacteria isolated from patients with urinary infections and from gastric juice of achlorhydric subjects**

We reported previously that 25 out of 38 microorganisms isolated from human subjects were able to nitrosate morpholine at neutral pH (Calmels *et al.*, 1985). We have now examined the nitrosation of morpholine by a total of 44 bacterial strains isolated either from patients with urinary infections or from gastric juice of patients with chronic atrophic gastritis or duodenal ulcers (Table 1). Procedures for collecting gastric juice and the study subjects have been described elsewhere (Bartsch *et al.*, 1984). Nitrate reductase activity was also determined in these bacterial isolates. Of the 14 strains from patients with urinary infections, all from the Enterobacteriaceae family, 12 catalysed the formation of NMOR, in the range 5-86 nmol NMOR/mg protein per h. Only nine of 30 gastric bacterial strains catalysed nitrosation of morpholine; two strains of *Neisseria mucosa* (Nos 84 113 and 84 31), however, had high nitrosation and nitrate reductase activities. All bacterial strains that catalysed the nitrosation reaction also had relatively high nitrate reductase activity.

**Table 1. Activities of some microorganisms isolated from patients with urinary infections and from gastric juice of human subjects to catalyse the nitrosation of morpholine at pH 7.2<sup>a</sup>**

Bacterial strain	Strain no.	Specific nitrosation activity (nmol NMOR/mg protein per h)	Nitrate reductase activity ( $\mu\text{g NO}_2/\text{mg protein per min}$ )
From gastric juice			
<i>Neisseria mucosae</i>	84 117	308	24.3
	85 31	238	18.7
	84 94	52	9.2
	84 100	5	5.8
12 other strains		ND	3.5 $\pm$ 1.4
<i>Neisseria perflava</i>	84 117	82	4.1
<i>Staphylococcus aureus</i>	85 27	ND	8.3
<i>Lactobacillus</i>	85 22, 85 29	ND	ND
<i>Pseudomonas maltophilia</i>	84 58	3.7	1.7
	84 148	ND	0.8
<i>Enterobacter cloacae</i>	84 121	1.0	3.8
	84 122	6.0	4.8
	84 146	6.0	4.4
<i>Escherichia coli</i>	84 153	ND	4.4
<i>Acinetobacter lwoffii</i>	84 147	ND	ND
<i>Corynebacteria saprophytes</i>	84 98, 84 135	ND	0.5
From urinary infections			
<i>Escherichia coli</i>	U 311 7	35	14.6
	U 307 2	86	19.8
	U 355 7	14	16
	U 340 15	ND	18
	U 347 4	17	13.8
	U 348 1	20	15.2
	U 348 9	22	15.6
	U 311 9	78	16.2
	<i>Klebsiella pneumoniae</i>	U 321 7	5
U 310 7		10	14
U 308 11		2	17
<i>Klebsiella oxytoca</i>	U 308 2	14	19.6
<i>Pseudomonas aeruginosa</i>	U 312 12	6	-
<i>Proteus morgani</i>	U 353 8	11	6.6

<sup>a</sup>ND, not detected; -, not determined

Bacteria were cultured overnight at 37°C in 100-ml stoppered bottles containing a rich infusion broth (Aer haemocult, Institut Pasteur) without further checking for aerobiosis. Assays for nitrosation were carried out in the presence of a number of bacteria equivalent to 1-5 mg protein per assay containing 0.1 mol/l Tris buffer pH 7.2, 25 mmol/l NaNO<sub>2</sub>, 25 mmol/l morpholine (final volume, 5 ml). After 1 h incubation at 37°C, the reaction was stopped by adding 20 ml 0.25 N sodium hydroxide to 1 ml of the reaction mixture, 500 ng/l *N*-nitrosopentylmethylamine were added as internal standard, and volatile nitrosamines were extracted in dichloromethane and analysed by gas chromatography-thermal energy analysis. Nitrate reductase activity was assayed by measuring the reduction of NaNO<sub>3</sub> to NaNO<sub>2</sub> with methyl viologen as the electron donor (McGregor *et al.*, 1974).

### Effects of culture conditions of *Escherichia coli* A10 on the induction of its nitrosation and nitrate reduction activities

In previous experiments (Calmels *et al.*, 1985) and in the assays shown in Table 1, bacteria were cultured in a nutrient-rich infusion broth. In order to study the effect of culture conditions on the catalysis of nitrosation, bacteria were cultured in a minimal medium (Table 2). Under these conditions, *E. coli* A10 did not exhibit nitrosation activity, although low nitrate reductase activity was detected. When nitrite or formate was added to the culture, no nitrosation activity was observed, although some residual nitrate reductase activity was seen. *E. coli* A10 cultured in minimal medium to which nitrate had been added alone, or with formate, exhibited high nitrosation and nitrate reductase activities. Therefore, nitrate appears to be essential for the induction of these two activities. However, when cysteine was present in culture medium containing nitrate, there was no induction of nitrosation or nitrate reduction. Since cysteine blocks the synthesis of hydrogenlyase and thus inhibits nitrate reductase (Azoulay *et al.*, 1969), this result suggests that nitrosation activity may be related to nitrate reductase.

**Table 2. Nitrosation and nitrate reductase activities of *E. coli* A10 cultured under different conditions<sup>a</sup>**

Culture medium	Specific nitrosation activity (nmol NMOR/mg protein per h)	Nitrate reductase activity ( $\mu\text{g NO}_2/\text{mg protein per min}$ )
Minimal medium (MM)	ND	8
MM + NaNO <sub>2</sub> (5 mmol/l)	ND	7.5
MM + HCOONa (10 mmol/l)	ND	7
MM + NaNO <sub>3</sub> (10 mmol/l)	146	32
MM + NaNO <sub>3</sub> (10 mmol/l) + HCOONa (10 mmol/l)	153	41
MM + cysteine (5 mmol/l)	ND	3.5
MM + NaNO <sub>3</sub> (10 mmol/l) + cysteine (5 mmol/l)	ND	2.2

<sup>a</sup>ND, not detected

Bacteria were cultured in stoppered 100-ml bottles at 37°C, without shaking or further checking for aerobiosis, in minimal medium composed of 3.07 g Na<sub>2</sub>HPO<sub>4</sub>, 1 g KH<sub>2</sub>PO<sub>4</sub>, 0.030 g MgSO<sub>4</sub> and 0.5 g NH<sub>4</sub>Cl in 1 litre of distilled water and 0.1% glucose was added after autoclaving.

### Nitrosation by *E. coli* A10 at different stages of the growth curve

In culture with minimal medium containing 0.1% glucose and 10 mmol/l sodium nitrate, the stationary growth phase was reached after 10 h of incubation at 37°C. Only resting cells of *E. coli* A10 were able to catalyse nitrosation of morpholine, and bacteria in the growing phase did not exhibit activity. Nitrate reductase activity, however, was observed in both the stationary and exponential phases. In contrast, both growing and resting cells of *E. coli* A10 cultured in rich infusion broth (Aer haemocult) were able to nitrosate morpholine.

### Nitrosation of morpholine by niridazole-resistant and chlorate-resistant mutants of *E. coli* A10

In order further to characterize the mechanism(s) by which bacteria catalyse nitrosation *in vitro*, several mutants deficient for either nitroreductase (*E. coli* A10 Nir<sup>R</sup>) or nitrate reductase/formate hydrogenlyase (*E. coli* A10 Chl<sup>R</sup>) were investigated. These mutant strains (prepared by Dr H.S. Rosenkranz and Dr E.C. McCoy, Case Western Reserve University, Cleveland, OH, USA) were grown on trypticase soya broth agar plates containing potassium chlorate (0.3%) or niridazole (40 µg/ml), and Chl<sup>R</sup> and Nir<sup>R</sup> mutants were selected after three days of anaerobic incubation at 37°C. The nitrosation activity of *E. coli* A10 Nir<sup>R</sup> cultured in rich infusion broth was not affected by loss of nitroreductase, but that of *E. coli* A10 Chl<sup>R</sup> disappeared completely. Single-step revertants prepared from *E. coli* A10 Chl<sup>R</sup>, in which the nitrate reductase/formate hydrogenlyase was restored, were found to catalyse nitrosation of morpholine *in vitro*.

Three different *E. coli* A10 Chl<sup>R</sup> revertants (cultured in minimal medium containing 0.1% lactose and 10 mmol/l sodium nitrate) were tested for their nitrosation and nitrate reductase activities (Table 3). In strains CNF 1001N<sub>1</sub>-1002N<sub>1,2,3</sub>-1003N<sub>1</sub>, the nitrosation activity strongly paralleled that for nitrate reductase/formate hydrogenlyase.

**Table 3. Activity of *E. coli* A10 Chl<sup>R</sup> and its revertants to catalyse the nitrosation of morpholine at pH 7.2 and to reduce nitrate<sup>a</sup>**

<i>E. coli</i> strain	Specific nitrosation activity (nmol NMOR/mg protein per h)	Nitrate reductase activity (µg NO <sub>2</sub> /mg protein per min)
A10	164 ± 25	23.4 ± 1.3
A10 Chl <sup>R</sup> mutants	ND	ND
A10 Chl <sup>R</sup> revertants		
CNF 1001 N <sub>1</sub>	42 ± 14	12.3 ± 0.5
CNF 1002 N <sub>1,2,3</sub>	140 ± 9	16.1 ± 0.5
CNF 1003 N <sub>1</sub>	173 ± 3	20.9 ± 1.3

<sup>a</sup>ND, not detected

Revertants were cultured in minimal medium containing 0.1% lactose and 10 mmol/l NaNO<sub>3</sub> and assayed three times for nitrosation of morpholine and reduction of nitrate, as described in the legend to Table 1.

### Effects of various metal ions, cofactors and nitrate on the *in vitro* nitrosation of morpholine by *E. coli* A10 and *N. mucosa* 84 113

The metal ions, Ag<sup>+</sup>, Pb<sup>2+</sup>, Hg<sup>2+</sup>, Mo<sup>2+</sup>, Al<sup>3+</sup> and Mg<sup>2+</sup>, when present at 2.5 mmol/l (as chlorides or sulphates) in reaction mixture containing nitrite, morpholine and bacteria cultured in rich medium, had no effect on the catalysis of nitrosation by *E. coli* A10 or *N. mucosa* 84 113. At the same concentration, Co<sup>2+</sup>, Cu<sup>2+</sup>, Ni<sup>2+</sup>, Fe<sup>2+</sup>, Mn<sup>2+</sup>, Zn<sup>2+</sup>, Se<sup>4+</sup>, Hg<sup>+</sup> and molybdic acid inhibited nitrosation reactions by 20-100%. Ca<sup>2+</sup>, Sn<sup>2+</sup> and Fe(CN)<sub>6</sub><sup>3-</sup> enhanced this activity by 40-76%. The presence of CN<sup>-</sup> (0.2 mmol/l) inhibited nitrosation by *E. coli* A10, which was partly restored by adding potassium ferricyanide (1 mmol/l).

The presence of either  $FAD^+$ ,  $FMN^+$ ,  $NAD(P)^+$  or riboflavin at concentrations of 0.5 mmol/l in the reaction mixture had no significant effect on bacterial nitrosation by either strain.  $NAD(P)H^+$  (0.5, 1, 2 mmol/l) strongly decreased nitrosation by *E. coli* A10, while, at the same concentrations, it had no effect on nitrosation by *N. mucosa* 84 113.

The presence of a 50-100 times lower concentration of nitrate than that of nitrite in the reaction mixture strongly inhibited in-vitro bacterial catalysis of nitrosation. Complete inhibition of nitrosation catalysis by *E. coli* A10 and *N. mucosa* 84 113 was induced by 0.25 and 0.5 mmol/l nitrate, respectively, in the reaction mixture. This suggests competition between nitrate and nitrite for the active site of the enzyme, or competition between the nitrosating enzyme and nitrate reductase for an electron donor.

### Conclusion

Bacterial strains belonging to the Enterobacteriaceae family, isolated from patients with urinary infections, were shown to catalyse the in-vitro nitrosation of morpholine at neutral pH. Similarly, some bacteria from the Neisseriaceae family, isolated from the gastric juice of achlorhydric subjects were found to have a particularly high activity for nitrosation of morpholine. With *E. coli* A10 and *N. mucosa* 84 113, the presence of nitrate was required in the culture medium to induce nitrosation activity; addition of low concentrations of nitrate totally inhibited the bacterial nitrosation activity. These results suggest that nitrate concentration plays a critical role in the kinetics of bacterial nitrosation. Increased levels of *N*-nitroso compounds have been demonstrated both in the urine of infected human subjects (Brooks *et al.*, 1972; Hicks *et al.*, 1977; Ohshima *et al.*, this volume) and in the gastric juice of patients with gastric achlorhydria (Reed *et al.*, 1981a; Stockbruger *et al.*, 1982; Böckler *et al.*, 1983; De Bernardinis *et al.*, 1983). Investigation of the role of bacteria and the nature of the enzymes possibly involved in the formation of these *N*-nitroso compounds in the human stomach and the urinary bladder thus remains an urgent task.

### Acknowledgements

We wish to thank Dr H.S. Rosenkranz and Dr E.C. McCoy (Case Western Reserve University, Cleveland, OH, USA) for preparing the *E. coli* A10 mutants. This research was undertaken during the tenure of a fellowship awarded to S. Calmels by the Ligue Nationale Francaise contre le Cancer.



# BACTERIALLY MEDIATED *N*-NITROSATION REACTIONS AND ENDOGENOUS FORMATION OF *N*-NITROSO COMPOUNDS

S.A. Leach<sup>1,3</sup>, A.R. Cook<sup>1</sup>, B.C. Challis<sup>2</sup>, M.J. Hill<sup>1</sup>  
& M.H. Thompson<sup>1</sup>

<sup>1</sup>PHLS Centre for Applied Microbiology and Research,  
Porton Down, Salisbury, Wiltshire SP4 0JH; and <sup>2</sup>Imperial College,  
Department of Chemistry, South Kensington, London SW7 2AY, UK

**Results are presented demonstrating some factors that affect the kinetics of bacterially mediated *N*-nitrosation reactions. Two groups of bacteria, differing in their nitrate/nitrite metabolism, are contrasted. These findings are discussed in relation to a role for bacteria in endogenous *N*-nitrosation reactions.**

A causative role for endogenously formed *N*-nitroso compounds in human carcinogenesis is suggested (i) by increased levels of the precursor nitrite consequent on bacterial overgrowth (a recognized risk factor for carcinogenesis [Caygill *et al.*, this volume]) and (ii) by the demonstration of the endogenous formation of *N*-nitroso compounds in humans both by direct measurement of these compounds as a group (Hicks *et al.*, 1977; Reed *et al.*, 1981a; Cook *et al.*, 1985) and by the study of the *N*-nitrosation of proline as a marker (Ohshima & Bartsch, 1981). Opinion is divided, however, as to how the endogenous formation of *N*-nitroso compounds is related to disease states (particularly achlorhydria) and bacterial overgrowth. Since chemical *N*-nitrosation occurs primarily at acid pH values, it is important to consider alternative pathways for *N*-nitrosation at or near neutral pH. The following data reassess bacterially mediated *N*-nitrosation reactions, since these represent the most likely alternative mechanism relevant to disease states. For a review of the literature on bacterially mediated nitrosation up to 1981, see Ralt and Tannenbaum (1981).

## Experimental

The methods used in the study involve a brief incubation of whole, washed, resting cells in a well-buffered solution of appropriate secondary amine and nitrite, as described previously (Leach *et al.*, 1985a), with analysis of *N*-nitrosamine formation by gas chromatography-thermal energy analysis. The usual checks were made for artefactual *N*-nitrosamine formation, and background levels attributed to concurrent chemical nitrosation were subtracted.

The activities of the various organisms (mostly clinical isolates) investigated in the *N*-nitrosation assay are shown in Table 1. The bacteria divide into two groups dependent on their ability to *N*-nitrosate morpholine and to reduce nitrite to either gaseous oxides of nitrogen or nitrogen gas (i.e., denitrifiers) (Leach *et al.*, 1986a,b).

<sup>3</sup> To whom correspondence should be addressed

**Table 1.** Rates of bacterial catalysis of the *N*-nitrosation of morpholine at neutral pH values

Non-denitrifying species	Strain <sup>a</sup>	Nitrosation rate <sup>b</sup>	Denitrifying species	Strain <sup>a</sup>	Nitrosation rate <sup>b</sup>
<i>Escherichia coli</i>	BM1056 F	0;55	<i>Pseudomonas aeruginosa</i> <sup>c</sup>	BM1030 G	0-24 000
	BM1023 B	39;44;60 <sup>c</sup> ;90 <sup>c</sup>		BM1035 G	2 000-8 000
	BM1042 B	26;14		BM1233(4) G	50-1 270
	BM1033 F	0;0	<i>Bacillus licheniformis</i> <sup>c</sup>	NCTC 8721 T	340-2 900
	BM1047 F	0			
	BM1046 G	0			
	BM1018 F	0			
	BM1052 -	76	<i>Neisseria</i> spp. <sup>c</sup>	2-1 0	720-1 890
		3-1 0		240	
		1-1 0		420-900	
<i>Streptococcus</i> sp.	6 G	0	2-2 0	260	
<i>Veillonella</i> sp.	2V G	0	<i>Alcaligenes faecalis</i> <sup>c</sup>	NCTC 415 T	1 310-3 280
<i>Veillonella</i> sp.	24 G	0			
<i>Bifidobacterium</i> sp.	10 G	0			
<i>Clostridium</i> sp.	8 G	0			
<i>Clostridium</i> <i>perfringens</i> <sup>c</sup>	F	0			
<i>Pseudomonas</i> <i>fluorescens</i> <sup>c</sup>	NCDO 10038 T	0			
<i>Acinetobacter calcoaceticus</i> <sup>c</sup>	NCTC 7844 T	0			
<i>Neisseria</i> sp. <sup>c</sup>	A1078 0	0			

<sup>a</sup>Source of bacteria: F, faeces or colonic mucosa; G, gastric; O, oral; B, urinary-tract infections. T, type of strain

<sup>b</sup>Nitrosation rate: nmol *N*-nitrosomorpholine/mg protein per h. Assays were carried out in 0.1 M phosphate buffer pH 7.2, 25 mM sodium nitrite, 8 mM morpholine, for 15 min at 37°C. Individual assay results are shown for non-denitrifiers; ranges are given for the many assays conducted on denitrifiers.

<sup>c</sup>16 mM morpholine, pH 8

The kinetics and pH dependence of the nitrosation reactions brought about by an *Escherichia coli* isolate (representative of the first group of non-denitrifying bacteria) clearly implicate some bacterial enzyme or enzyme system (Leach *et al.*, 1985a,b). The activities of these bacteria are in close agreement with those reported by other workers (Suzuki & Mitsuoka, 1984; Calmels *et al.*, 1985), but their small magnitude makes their clinical relevance questionable.

The denitrifying bacteria, by contrast, show much faster rates of reaction — typically ten to 100 times those of the best *E. coli* isolate. The denitrifying bacteria, however, require to be induced by prior anaerobic growth with either nitrate or nitrite as terminal electron acceptors (Table 2). This is again in marked contrast to the results obtained with the two *E. coli* isolates studied, the *N*-nitrosating abilities of which are suppressed by anaerobic growth with nitrate or nitrite.

**Table 2. Dependence of bacterial catalysis on the metabolic capability of the organism and its growth conditions**

Growth conditions <sup>a</sup>	Rates of nitrosation of morpholine <sup>b</sup>		
	<i>E. coli</i> BM1023	<i>E. coli</i> BM1056	<i>P. aeruginosa</i> BM1030
Anoxic	60	89	- <sup>c</sup>
Aerobic	33	23	6
Anaerobic + nitrate (40 mM)	0	3	7900; 5200
Anaerobic + nitrite (10 mM)	0	7	540; 7300

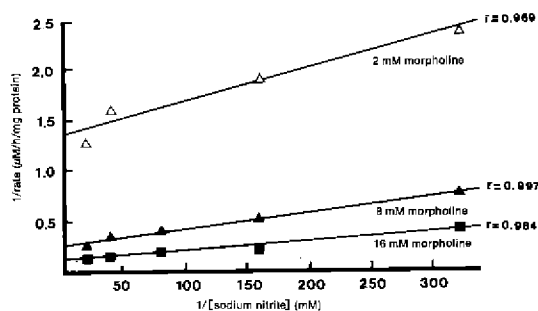
<sup>a</sup>Growth conditions: overnight in Tryptone soya broth, 37°C with additions as in Table 1

<sup>b</sup>Rates: nmol/mg protein per h. Assays carried out in 0.1 M phosphate buffer pH 8, 25 mM sodium nitrite, 16 mM morpholine for 15 min at 37°C

<sup>c</sup>*P. aeruginosa* is an obligate aerobe.

simple chemical kinetics of the form (1) rate =  $K$  [nitrite] [amine] or (2) rate =  $k$  [nitrite]<sup>2</sup> [amine] do not apply to the data. With high concentrations of nitrite, substrate inhibition is observed. The pH optimum for the *N*-nitrosation of each amine catalysed by *P. aeruginosa* was in the range 6-9 (morpholine, 8-8.5; pyrrolidine, 8-8.25; piperidine, 7.5-7.75), and their relative rates of reaction showed no obvious dependence on the basicity of the amine (Table 4).

**Fig. 1. Lineweaver-Burk plots for the *N*-nitrosation of morpholine mediated by *P. aeruginosa***



Assays performed for 15 min at 37°C in 0.1 M phosphate buffer pH 8, 0.22 mg/ml bacterial protein, using three concentrations of morpholine; *r*, correlation coefficient

Subsequent kinetic experiments used *P. aeruginosa* BM 1030, which gave qualitatively similar results to those obtained in earlier studies with *E. coli* isolates. Thus, the rate of nitrosation of both morpholine and *N*-methylpiperazine was linear over the first 45 min and was proportional to the cell concentration used (measured as mg protein/ml). Further, cell suspension supernatants and sonicated cells showed no significant activity in the assay (Table 3). Lineweaver-Burk plots (i.e., 1/rate vs. 1/[substrate]) of the kinetic data show good best-fit linear regression lines ( $p < 0.05 - 0.001$ ) with both morpholine and nitrite (Fig. 1). Thus,

## Conclusions

The form of the kinetic data (i.e., classical Michaelis-Menten kinetics, substrate inhibition with nitrite, dependence on whole, live bacterial cells and similar rates of reaction for amines of different basicity) suggest a mechanism of bacterially catalysed *N*-nitrosation reaction(s) for secondary amines that is mediated by an enzyme or enzyme system, the precise configuration of which has yet to be clearly elucidated. The most likely possibilities during denitrification are (1) the production of a reactive enzyme-nitrogen oxide intermediate which reacts directly with

**Table 3.** *N*-Nitrosating activities of cells and cell fractions of *P. aeruginosa* BM1030

Cell treatment	Yield <i>N</i> -nitrosomorpholine/ 30 min (ppm) <sup>a</sup>
Fresh cells (0.45 mg/ml protein) suspension	266
Cells pelleted from suspension (resuspended) <sup>b</sup>	167
Cell suspension supernatant <sup>b</sup>	1.7
Sonicated cell suspension <sup>c</sup>	68
Sonicated suspension pellet (resuspended) <sup>b</sup>	30
Sonicated suspension supernatant <sup>b</sup>	2.1

<sup>a</sup> Assay performed in 0.1 M phosphate buffer pH 8, 16 mM morpholine, 25 mM sodium nitrite at 37°C

<sup>b</sup> Centrifugation at 9000 × g for 5 min

<sup>c</sup> Cells sonicated in ice at maximum power in short bursts (30 sec) for total of 7 min

**Table 4.** Nitrosation catalysed by *P. aeruginosa* BM1030 for amines of differing basicity

Amine	pKa	Nitrosation rate relative to that of morpholine <sup>a</sup>
Methyl piperazine	5.6; 9.8	0.8; 1.18
Morpholine	8.7	1.0
Piperidine	11.2	0.3
Pyrrolidine	11.27	0.2; 0.8

<sup>a</sup> Relative nitrosation rate: rate for morpholine determined at each assay, and taken to be 1 to account for variability in cell preparations. Assays carried out in 0.1 M phosphate buffer pH 8, 25 mM sodium nitrite, 16 mM morpholine for 15 min at 37°C

of a flora *per se*. This suggestion may explain why only some achlorhydrics eventually develop cancer (Caygill *et al.*, this volume). A much higher incidence of cancer might have been expected if rapid *N*-nitrosation reactions catalysed at neutral pH values were a more prevalent phenomenon among bacterial species.

#### Acknowledgement

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secondary amines or (2) the reduction of nitrite to produce gaseous oxides of nitrogen, which then react chemically to form *N*-nitroso compounds. Since nitrite dioxide is an unlikely bacterial product, its formation would necessitate, firstly, the production of free nitric oxide (a well-known nitrosating species) by the bacteria and would also be dependent on the availability of oxygen as a further substrate in the reaction. Our results (unpublished) regarding the oxygen sensitivity of these reactions so far suggest this latter mechanism to be unlikely. Furthermore, it is not clear that NO itself is a usual free intermediate of bacterial nitrite reduction. A careful study of the role of oxygen in these reactions should resolve this question.

The above data also show that the *N*-nitrosating ability of bacteria is markedly sensitive to both the metabolic capabilities of the organism concerned and its culture conditions. This suggests that an important factor for endogenous *N*-nitrosation reactions in chronic bacterial infection is the metabolic capability of the particular colonizing organism(s) and their growth conditions rather than the development

# IN-VITRO PRODUCTION OF NITROSAMINES BY BACTERIA ISOLATED FROM THE OPERATED STOMACH

C.M. O'Donnell<sup>1,2,4</sup>, C. Edwards<sup>2</sup>, G.D. Corcoran<sup>1</sup>, J. Ware<sup>3</sup> & P.R. Edwards<sup>1</sup>

<sup>1</sup>Department of Surgery and <sup>2</sup>Department of Microbiology,  
University of Liverpool, Box 147, Liverpool L69 3BX, UK; and  
<sup>3</sup>Department of Surgery, Karolinska Institute, Stockholm, Sweden

Evidence is presented of in-vitro catalysis of nitrosation by organisms isolated from the hypoacidic operated stomach. Subjects taking part in a prospective study of potential premalignancy after benign ulcer surgery underwent endoscopy, and samples of gastric juice were obtained aseptically. The organisms present were identified using the API system and tested for their ability to catalyse the nitrosation of the secondary amine, morpholine, at neutral pH and 37°C. Four of the five species tested were found to be capable of the catalysis. Cellular disruption and denaturation of protein abolished the catalytic ability, suggesting that the catalysis is mediated by an enzymic system. Osmotic shock experiments indicate that the enzyme site may be on the inner membrane.

An increased risk of primary gastric malignancy 20-25 years after operation for benign ulcer (Stalsburg & Taksdal, 1971; Haenszel & Correa, 1975) has been correlated with the hypoacidity caused by the ulcer operation and with a bacterial overgrowth (Muscroft *et al.*, 1981); the possible link may be *N*-nitroso compound formation from dietary precursors dependent upon bacterial catalysis (Hawksworth & Hill, 1971). Recent studies have lent support to this theory, showing that nitrosation in the presence of bacteria at neutral pH followed Michaelis-Menten kinetics. We have studied the in-vitro catalysis of nitrosation effected by bacteria isolated from the stomachs of individuals who had previously undergone surgery for benign ulcer.

## Patients and isolation of bacteria

Twenty-two study subjects (16 men, six women; operation interval, 16.6 years; 58.5 ± 2.37 years of age) were matched for age and sex with 21 control subjects (13 men, eight women; 59.5 ± 3.4 years) undergoing routine endoscopy for dyspepsia.

Gastric aspirate was obtained aseptically at endoscopy using the method of Muscroft *et al.* (1981). The endoscope was cleaned before sampling with 0.015% chlorhexidine gluconate in 0.15% cetrimide and then rinsed thoroughly with sterile, distilled water. Routine culture of the water aspirated following cleaning of the instrument showed that it was consistently sterile, confirming the asepsis of this technique. The technical difficulties involved in the maintenance of a completely anaerobic environment prevented the scope of the study being extended to include anaerobic and microaerophilic organisms. Aerobic and facultatively anaerobic organisms were identified using the API 20E, API Staph and API Strep systems.

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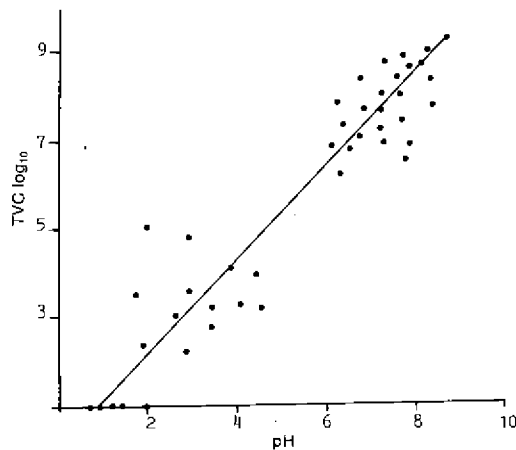
<sup>4</sup> To whom correspondence should be addressed

Aspirate (0.5 ml) was inoculated into nutrient broth transport medium (Oxoid) prior to serial dilution in Ringer's solution (Oxoid) and incubated at 37°C on nutrient agar and McConkey agar for 48 h. Following incubation, discrete colonies were examined morphologically by Gram staining, oxidase and catalase tests and then plated onto blood agar for identification using the API system. The total viable count (TVC) was calculated for each species and then expressed as a percentage of the TVC for the aspirate as a whole.

The bacterial flora of the human stomach with no associated dyspepsia or lesion reflects the organisms that have been swallowed (Drasar *et al.*, 1969). At low pH, gastric juice is sterile, but following a meal the acid is neutralized and bacterial counts increase (Drasar *et al.*, 1969; Keighley *et al.*, 1984). Following an ulcer operation, a state of hypoacidity prevails, which is associated with periodic bacterial overgrowth (Keighley *et al.*, 1984). Our results in control subjects and individuals who had undergone ulcer surgery more than 15 years previously corroborate these reports.

Sixteen different species were isolated in the study. Numbers of bacteria were shown to be dependent on pH (Fig. 1). At any one pH value, the numbers were similar in the operated and nonoperated groups, but the species present differed. In the nonoperated group, *Streptococcus* spp. represented on average 52.7% of the population and *Staphylococcus* spp. formed 24.4%, the remaining species being similarly representative of the oral flora, e.g., *Lactobacillus* and *Actinomyces* spp.

**Fig. 1. Relationship between total viable count (TVC) of bacteria and pH of gastric juice**



was decided to confine the study of nitrosation to the enterobacteria, since the risk presented by the nitrosating ability of the other species would be dependent upon bacterial numbers as determined by pH rather than by a history of gastric surgery. Five species of enterobacteria were tested for their ability to catalyse nitrosation of the secondary amine, morpholine.

In the operated group, organisms more commonly found lower in the gut were isolated in addition to representatives of the oral flora. *Escherichia coli* occurred in 38.1% patients, in numbers ranging from  $4 \times 10^6$  —  $5.95 \times 10^8$  organisms/ml, which represented 27.4% of the whole population of this group. *Enterobacter* spp were present in 19.0% of the aspirates and formed on average 22.2% of the population in which they occurred. *Klebsiella pneumoniae* was isolated from 14.2% of patients with previous gastric surgery.

The difference in bacterial species between the two groups, which cannot be attributed to the pH value of the aspirate or the composition of the oral flora, is the presence of enterobacteria in the operated group. In view of this, it

### Bacterial nitrosation studies

The nitrosation reaction was carried out using a modification of the methods of Suzuki and Mitsuoka (1984) and Kunisaki and Hayashi (1979). Reaction mixture (20 ml) containing 11 mM morpholine, 25 mM nitrite and 6 mg/ml of cells was incubated at 37°C in a shaking water-bath at 200 rev/min for 60 min. *N*-Nitrosomorpholine (NMOR) produced in the reaction was extracted with an equal volume of dichloromethane for 3 min using a vortex mixer and quantified by gas-liquid chromatography linked to a Thermal Energy Analyzer (TEA 502 Thermo Electron). All experiments were performed with a control containing no cells to allow correction for chemical nitrosation. Samples were analysed within 1 h of extraction; samples awaiting analysis were kept on ice in the dark.

The results are shown in Table 1. Four of the five species tested could catalyse nitrosation. The values compare well with the range of results obtained by other workers using the same species and allowing for intrastain differences (Calmels *et al.*, 1985). Rates of nitrosation were found to be directly proportional to the protein concentration used. Heating the cells to 80°C for 10 min and subjecting the cell suspension to sonication abolished the ability of the cells to catalyse nitrosation.

**Table 1. Rate of production of NMOR at pH 7 and 37°C by organisms isolated from operated stomachs**

Organism	Rate of nitrosation <sup>a</sup> (nmol/mg protein per h)	
	A	B
<i>E. coli</i>	8.7	2.3
<i>E. coli</i> II	6.6	1.9
<i>E. coli</i> II	13.3	3.0
<i>Enterobacter aerogenes</i>	0.3	1.11
<i>Enterobacter agglomerans</i>	3.4	1.9
<i>Klebsiella pneumoniae</i>	5.6	2.0

<sup>a</sup>The values in column B give the amount of NMOR formed per ml per hour in the absence of cells; these values were subtracted from the results in the presence of cells to give the values in column A. Cells were grown in a modified tryptone soya broth. Cultures were grown aerobically at 37°C in an orbital incubator at 200 rev/min (500 ml in a 2-litre conical flask). Overnight cultures of cells were harvested by refrigerated centrifugation at 15 000 × g for 10 min, washed with 0.1 M phosphate buffer at pH 7 and resuspended to give a final protein concentration of about 6 mg/ml in the reaction mixture. All the above procedures were carried out at 0-4°C.

oxygen limitation. This would suggest that the catalytic action is an energy-dependent process, since the energy yield from anaerobic metabolism is less than that of aerobic metabolism. Taken in conjunction with the abolition of catalysis following cellular disruption and the implication from the osmotic shock experiments that the site of catalysis is on the inner membrane, it is suggested that the catalysis of nitrosation is linked to the membrane-bound respiratory chains.

In an attempt to locate the site of the observed catalysis, cells of *E. coli* B were shocked osmotically using the method of Nossal and Heppel (1966). This did not decrease viability; shocked cells showed 63% viability compared with a 62% viability of non-shocked cells after harvesting and washing. Treating the cells in this way prior to nitrosation did not affect the rate of catalysis observed, suggesting that the protein responsible for the catalysis may be located in the inner membrane or within the cytoplasm. The former would seem the more likely location, since cell disruption destroys the nitrosating ability of the cells.

Cells grown anaerobically showed a depression of their catalytic abilities (data not shown). Limiting the oxygen available to aerobically grown cells for the duration of the nitrosation reaction decreased their production of NMOR, the level of reduction being dependent upon the degree of

Inclusion of nitrite in the growth medium had a variable effect upon the rates of nitrosation, depending on the species. Under aerobic conditions, a stimulatory effect was noted in *K. pneumoniae* and *E. coli* B, while other species were inhibited by the presence of nitrite during growth. The reaction of nitrite with the cytochrome oxidase *d* component of the bacterial respiratory chain has been well documented (Meyer, 1973; Ingledew & Poole, 1984); however, we could not demonstrate a relationship between either cytochrome *d* content or the reduced cytochrome *d*-nitrite interaction with the nitrosation capacity of the cells (data not shown).

Epidemiological evidence might support an association between acid-reducing operations and the role of *N*-nitroso compounds in the causation of human gastric cancer (Stalsburg & Taksdal, 1971). This effect may be more significant when the stomach is not resected, as with vagotomy procedures (Watt *et al.*, 1984). However, a recently completed epidemiological study by our group was unable to confirm any increased risk of gastric cancer following gastrectomy or gastrojejunostomy 20 years previously (Corcoran *et al.*, 1985).

Although the link between hypoacidity after ulcer surgery, bacterial overgrowth and gastric malignancy may not have been proven, the results of this work on human gastric aspirate support previously reported data on the catalysis of nitrosamine formation by bacteria from other human sources at neutral pH (Calmels *et al.*, 1985). Thus, endogenous production of *N*-nitroso compounds, mediated by the normal enteric flora, may present a significant source of carcinogen in neutral areas throughout the gut. These results, obtained *in vitro*, give no indication of the potential magnitude of the *in-vivo* risks.

#### Acknowledgement

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## **MEASUREMENTS OF EXPOSURE**

# EXCRETION OF METHYLATED NUCLEIC ACID BASES AS AN INDICATOR OF EXPOSURE TO NITROSATABLE DRUGS

D.E.G. Shuker<sup>1</sup>, E. Bailey & P.B. Farmer<sup>2</sup>

MRC Toxicology Unit, Medical Research Council Laboratories,  
Woodmansterne Road, Carshalton, Surrey SM5 4EF, UK

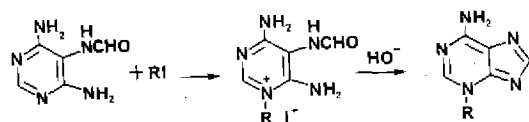
Urinary excretion of 3-methyladenine (3-meA) may be a valuable monitor of exposure to methylating carcinogens. In rats, the compound is known to be liberated from DNA following its formation, is not metabolized and is excreted intact in the urine. Studies with rats administered d<sub>3</sub>-methylating agents [e.g., a mixture of d<sub>6</sub>-aminopyrine (d<sub>6</sub>-AP) and nitrite which liberates d<sub>3</sub>-N-nitrosodimethylamine (NDMA) intragastrically] have confirmed that d<sub>3</sub>-3-meA is excreted in a dose-related manner. Although low levels of 'background' 3-meA have been found in rat and human urine, these are about 1000-fold lower than those of 7-methylguanine (7-meG). Thus, despite the fact that 3-meA is known to be formed at 10% of the level of 7-MeG in DNA following exposure of rats to methylating agents, the low 'background' that we have observed indicates that urinary 3-meA should be a more sensitive indicator of methylation than 7-meG.

Exposure of nucleic acids to methylating carcinogens results in the formation of 7-meG and 3-meA residues, which are released from the nucleic acids and excreted intact in the urine (Craddock & Magee, 1967; Hanski & Lawley, 1985). Exposure monitoring methods using measurements of urinary 7-meG are limited in sensitivity owing to the background levels of this methylated base (Shuker *et al.*, 1984a,b; Farmer *et al.*, 1986); we have therefore explored the exposure monitoring potential of determinations of 3-meA, for which no urinary background levels have been reported.

## Metabolism of 3-meA

If urinary 3-meA is to be used as a monitor of exposure to methylating agents, it must not be further metabolized following its release from the nucleic acids. The metabolism of labelled 3-meA has therefore been studied in rats. An unambiguous synthesis of 3-meA (Denayer, 1962) was used to prepare the tritiated and deuterated analogues in good yield as crystalline compounds (Fig. 1).

Fig. 1. Synthesis of tritiated (R = -CH<sub>2</sub>T) and deuterated (R = -CD<sub>3</sub>) 3-meA



<sup>3</sup>H-3-MeA (1.6 μCi, 0.37 mg) was administered to female rats (200-213 g, LAC:P strain) by gavage or intraperitoneal injection (two animals in each group). In both cases, 92-96% of the dose was excreted in the urine in the first 24 h after dosing, and a

<sup>1</sup>Present address: International Agency for Research on Cancer, 150 cours Albert-Thomas, 69372 Lyon Cedex 08, France

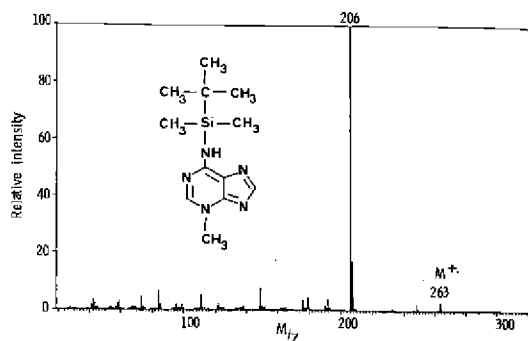
<sup>2</sup>To whom correspondence should be addressed

further 1-2% was excreted in the successive 24-h period. Analysis of the urine by thin-layer chromatography showed a single radioactive spot which co-chromatographed with authentic 3-meA. These results suggest that 3-meA, like 7-meG, is resistant to purine catabolic enzymes and is excreted unchanged *via* the urine.

### Isolation and analysis of 3-meA from urine

Full details of the isolation and analysis procedure will be described elsewhere. In brief, 1-ml samples of rat urine were chromatographed on a column of XAD-2 resin in 0.001 N hydrochloric acid, and the fraction that would contain 3-meA was purified further by high-performance liquid chromatography on an Ultrasphere ODS column (15 cm  $\times$  4.6 mm), using a gradient of 0.5% heptafluorobutyric acid and methanol. The 3-meA fraction was blown to dryness under nitrogen and derivatized for gas chromatography-mass spectrometry. In our hands, 3-meA has proved remarkably resistant to derivatization by standard reagents; however, we have found that the relatively new silylating reagent *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide readily affords the corresponding mono-*tert*-butyldimethylsilyl (TBDMS) derivative of 3-meA. 3-MeA-TBDMS has good gas chromatographic properties on a well-deactivated capillary column (OV-1701 or SE-52). The electron impact mass spectrum shows only a small  $M^+$  ion at  $m/z$  263 (2.8%) but a strong characteristic ( $M-C_4H_9$ )<sup>+</sup> ion at  $m/z$  206 (100%), which can be used for selected-ion monitoring (Fig. 2). 3-MeA can be quantified using a  $d_3$ -labelled internal standard (500 ng added to the initial 1-ml urine sample) and by monitoring the  $m/z$  209 ion;  $d_3$ -3-meA is quantified similarly using 3-meA as internal standard. Linear calibration lines were obtained for the analysis of urine aliquots containing increasing amounts (0-200 ng) of spiked 3-meA, and satisfactory reproducibility was obtained for analysis of replicate ( $n=6$ ) samples.

**Fig. 2.** Electron impact mass spectrum of the mono-*tert*-butyldimethylsilyl derivative of 3-meA



carcinogen. Although there has been no report of the natural occurrence of 3-meA as a base in DNA or RNA, or in rat or human urine, we chose again to use labelled methylating agents to assess the potential of 3-meA determinations for exposure monitoring.

AP is known to be a hepatocarcinogen in the presence of nitrite (Taylor & Lijinsky, 1975) and is thought to produce NDMA *via* intragastric nitrosation. Gombar *et al.* (1983) have shown that  $^{14}C$ -7-meG is excreted by rats following treatment by gavage with a mixture

### Formation of 3-meA *in vivo*

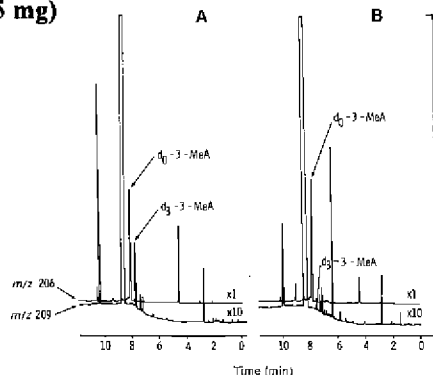
In our earlier work, on estimating 7-meG, we chose to study methylation arising from stable isotope labelled ( $d_3$ ) carcinogens (Shuker *et al.*, 1984a, b). 7-MeG exists at significant levels in rat (Mandel *et al.*, 1966) and human (Weissman *et al.*, 1957a) urine, and it was therefore impractical to measure low levels of carcinogen-induced methylation from determinations of urinary 7-meG. The use of deuterated methylating agents allowed unequivocal identification of 7-meG arising from the

of  $^{14}\text{C}$ -AP and sodium nitrite. AP labelled with six deuterium atoms in its  $\text{N}(\text{CH}_3)_2$  group ( $\text{d}_6$ -AP) was also shown to produce a dose-dependent excretion of  $\text{d}_3$ -7-meG in rat urine when administered by gavage in the presence of nitrite (Shuker *et al.*, 1984b; Farmer *et al.*, 1986). A similar dosing protocol was therefore adopted for this study.

Female rats (150-200 g, LAC:P strain) were treated by gavage with  $\text{d}_6$ -AP (5 mg/rat) and varying doses of sodium nitrite (0-10 mg/rat). The animals were housed in polycarbonate metabolism cages and allowed food (MRC 41B powdered diet) and water *ad libitum*. Urine was collected over 24-h periods, frozen and later standardized in volume to 10 ml; 1-ml aliquots were used for determination of  $\text{d}_3$ -3-meA using  $\text{d}_0$ -3-meA (500 ng) as internal standard. Parallel analyses were carried out with no internal standard, in order to assess the amount of unlabelled 3-meA in the rat urines.

Examples of selected ion monitor traces ( $m/z$  206 for  $\text{d}_0$ -3-meA and  $m/z$  209 for  $\text{d}_3$ -3-meA) for a rat dosed only with  $\text{d}_6$ -AP (5 mg) and for a rat dosed with  $\text{d}_6$ -AP (5 mg) and sodium nitrite (10 mg) are given in Figure 3. Addition of nitrite to the drug increased significantly the excretion of  $\text{d}_3$ -3-meA. Preliminary values for the excretion of  $\text{d}_3$ -3-meA in the first 24 h after dosing are:  $\text{d}_6$ -AP (5 mg), 42 ng;  $\text{d}_6$ -AP (5 mg) plus  $\text{NaNO}_2$  (1 mg), 130 ng;  $\text{d}_6$ -AP (5 mg) plus  $\text{NaNO}_2$  (2.5 mg), 190 ng;  $\text{d}_6$ -AP (5 mg) plus  $\text{NaNO}_2$  (5 mg), 322 ng; and  $\text{d}_6$ -AP (5 mg) plus  $\text{NaNO}_2$  (10 mg), 436 ng. (The significance of the result for  $\text{d}_6$ -AP alone is tentative, as the observed selected ion monitor peak is due in part to the isotopic contribution from the internal standard peak at  $m/z$  206.)

**Fig. 3. Selected ion monitor trace ( $m/z$  206 and  $m/z$  209) for urinary 3-meA for (A) a rat (171 g) treated with  $\text{d}_6$ -AP (5 mg) and sodium nitrite (10 mg), and (B) a rat (155 g) treated with  $\text{d}_6$ -AP (5 mg)**



and nitrite proves that the drug is converted to a methylating agent by the action of nitrite, which transfers all of its hydrogen atoms to the nucleophilic substrate. This is known to be the case for  $\text{d}_6$ -NDMA (Lijinsky *et al.*, 1968), which is postulated to be the product formed from AP and nitrite.

In parallel experiments in which no 3-meA was added to the urine samples, a small peak corresponding to about 200 ng/24 h was seen at the retention time of 3-meA; its identity is currently being studied. Similarly, control human urines (10-ml aliquots;  $n = 10$ ) appear to contain a low background level of 3-meA (9.6  $\mu\text{g}/24$  h) corresponding to  $1.2 \times 10^{-3}$  for rats (Shuker *et al.*, 1984a) and  $1.4 \times 10^{-3}$  for humans (Weissman *et al.*, 1957b) of the published values for 7-meG excretion. 3-meA is known to be produced in DNA following exposure to a methylating agent at about 10% of the level of 7-meG. Consequently, determination of 3-meA is potentially about 100 times more sensitive, relative to background, than determination of 7-meG.

The fact that trideuterated 3-meA is formed following exposure of rats to  $\text{d}_6$ -AP

The greater sensitivity for detecting methylation relative to background (compared to 7-meG), and our success in demonstrating the production of a methylating agent from AP, indicate that measurement of urinary 3-meA may be a useful method for monitoring potentially nitrosatable drugs for alkylating ability. Such studies are currently in progress in our laboratory, and application of this technology to human material is also planned.

**Acknowledgements**

We would like to thank Angela Parry for her expert help with the analytical determinations and John Lamb for gas chromatographic-mass spectrometric measurements.

# NITROSAMINE MEASUREMENTS IN AMBIENT AIR OF AN INDUSTRIAL AREA IN AUSTRIA

B. Spiegelhalder & R. Preussmann

*Institute for Toxicology, German Cancer Research Center,  
Heidelberg, Federal Republic of Germany*

The area of Linz (Oberösterreich) is the most heavily polluted region in Austria, due to its chemical and steel industry. In 1981, a survey of volatile nitrosamines in ambient air performed by a local laboratory revealed levels of up to  $5.45 \mu\text{g}/\text{m}^3$ . This instigated the setting up of a systematic nitrosamine monitoring programme from February 1983 to May 1984, during which the validity of the analytical procedures was determined. A total of 363 air samples was collected over 200 days at 16 different locations in and around Linz. About 6% of the samples showed low nitrosamine contamination, with levels between  $0.01$  and  $0.04 \mu\text{g}/\text{m}^3$  of *N*-nitrosodimethylamine (NDMA), *N*-nitrosodiethylamine (NDEA), and *N*-nitrosomorpholine (NMOR). The lower limit of detection was  $0.005 \mu\text{g}/\text{m}^3$ . It was not possible to confirm these low concentrations by high-resolution mass spectrometry. In some samples, thermal energy analyser-responsive material was observed, which may be due to the occurrence of *C*-nitro compounds.

In 1981, a survey of volatile nitrosamines in ambient air in the area of Linz (Oberösterreich), a heavily polluted region in Austria, revealed levels of nitrosamines up to  $5.45 \mu\text{g}/\text{m}^3$ . Since such high concentrations of nitrosamine would represent a considerable risk for the population living in this area, it was necessary to evaluate the emission situation by a systematic survey using validated analytical procedures.

## Sampling strategies

A total of 16 sampling places were selected in the area of Linz (Fig. 1). After a testing period of four months, regular sampling started in June 1983 for one year. An average of five sampling days per month was randomly selected in three different sampling places. The sampling conditions were as follows: separate 8-h samples were collected during day and night; the volume collected was usually 400-500 l; humidity and temperature were measured before and after sampling; meteorological data and the concentration of nitrogen oxides in air were supplied by the nearest air monitoring station of the state of Oberösterreich; sampling was performed by Dr Kronraff (Department of Radiology, National Hygiene Institute, Linz).

## Materials and methods

Ambient *N*-nitrosamines were collected at a height of 1.8 m with adsorption air collectors (ThermoSorb/N, Thermo Electron) connected to a constant flow air pump (HSG-12, 0.5-12 l/min, ILS Co.). The flow rate was about 1 l/min. Elution and determination of *N*-nitrosamines were done as described previously (Spiegelhalder & Preussmann, 1983b).

**Fig. 1. Sampling sites in the area of Linz-Steyregg**



1. 24 — Tower 415\*; 2. Hauserhof — roof 401\*; 3. School centre 416\*; 4. Heating plant; 5. Entrance, coke and steel plant (VOEST); 6. Steyregg village, point II 417\*; 7. ORF centre (Austrian radio station) 414\*; 8. Wagner-Jauregg Hospital, Niedernhart; 9. Steyregg village, point I; 10. BBSUA (National Hygiene Institute), Dörflingerstrasse; 11. Chemie Linz, Brunnenstation; 12. Plesching — suburban area; 13. ESG-Mühlbach — suburban area; 14. Steyregg village, point III, Birkhofer; 15. Steyregg village, point IV, Buchner; 16. Kleinmünchen 412\* — suburban area

\* Number in the State 'automatic air monitoring network'

### Results and discussion

A total of 363 air samples were collected between 10 February 1983 and 26 May 1984 and analysed for volatile *N*-nitrosamines. In 78, low levels of organic NO-releasing compounds were identified as volatile *N*-nitrosamines by gas chromatography-chemiluminescence detection (lower limit of detection, about  $0.005 \mu\text{g}/\text{m}^3$ ). NDMA was found in 54 samples and NDEA in 45 samples; 16 air samples contained both nitrosamines. NMOR was detected in only two samples. The concentrations of the observed *N*-nitrosamines were between 0.004 and  $0.04 \mu\text{g}/\text{m}^3$ ; these low levels could not be confirmed by high-resolution mass spectrometry.

The most important finding of this study is that the highest observed level was not greater than  $0.04 \mu\text{g}/\text{m}^3$ . Results of an earlier study (Beger, 1981), showing NDMA concentrations 100 times higher than those observed in this investigation, could therefore not be confirmed. The frequencies of two concentration ranges of NDMA and NDEA in different sampling places are given in Table 1. *N*-Nitrosamines were detected more often at sampling places 1, 3, 5, 7, 10, 11 and 14 than in the other places.

**Table 1. Frequencies of samples containing NDMA and NDEA in two concentration ranges related to sampling location**

Sampling location <sup>a</sup>	No. of samples	Number of samples containing NDMA/NDEA	
		Up to $0.01 \mu\text{g}/\text{m}^3$	$> 0.01-0.04 \mu\text{g}/\text{m}^3$
1	20	4	3
2	21	0	1
3	25	5	0
4	15	1	1
5	26	9	0
6	35	5	1
7	19	4	3
8	24	8	1
9	31	6	1
10	38	7	3
11	23	3	3
12	15	0	0
13	12	0	0
14	24	5	5
15	19	4	0
16	16	0	0
Total	363	61	22

<sup>a</sup>See Figure 1

500°C). This behaviour indicates the presence of aliphatic *C*-nitro compounds. The observed levels were too low for further identification.

No effect of meteorological conditions on the occurrence of *N*-nitrosamines was observed. Although high concentrations of nitrogen dioxide seem to correlate with a higher frequency of positive *N*-nitrosamine levels, the absolute number of observations was too small for a final evaluation. Table 2 shows the distribution of frequencies of *N*-nitrosamine levels broken down by nitrogen dioxide concentration.

In a number of chromatograms, peaks with retention times different from those of known volatile *N*-nitrosamines were observed, most of which appeared before NDMA; some had retention times similar to those of NDMA, NDEA and *N*-nitrosodipropylamine. Further investigations showed, however, that the detector signal intensity of these peaks increased with elevated pyrolysis temperature ( $650^\circ\text{C}$  instead of



**Table 2. Frequencies of samples containing NDMA and NDEA at different concentration ranges in relation to nitrogen dioxide levels**

Nitrogen dioxide (mg/m <sup>3</sup> )	No. of samples	Number of samples containing NDMA/NDEA			
		Below detection limit	< 0.01 µg/m <sup>3</sup>	Up to 0.02 µg/m <sup>3</sup>	Up to 0.04 µg/m <sup>3</sup>
up to 0.18	2	1	-	1	-
0.16	3	0	-	1	2
0.14	1	0	-	1	-
0.12	12	7	2	2	1
0.10	19	15	3	1	-
0.08	42	30	7	3	2
0.06	65	39	23	3	-
0.04	91	68	18	4	1
0.02	35	27	7	1	-

In addition to air measurements, dust samples were also analysed for *N*-nitrosamines. Dust was collected as atmospheric deposition at monthly intervals, and also using a high-volume dust filter device. Both *N*-nitrosamines and *C*-nitro compounds were detected at levels of up to 0.3 ppb. Due to the collection method, it is not possible to calculate the corresponding air concentration. The two dust samples collected on the filter contained NDMA at concentrations of up to 180 ppb; due to the large air volume collected, this corresponds to an air concentration of 0.00001 µg/m<sup>3</sup>.

### Conclusions

*N*-Nitrosamines occur in the ambient air in the area of Linz-Steyregg at concentrations below 0.4 µg/m<sup>3</sup>; only 6% out of 363 samples contained > 0.01 µg/m<sup>3</sup>. Thus, the presence of high levels of precursors (an amine-producing chemical plant, a coke plant, high nitrogen oxide emissions) does not necessarily give rise to extensive *N*-nitrosamine formation. Under the most unfavourable conditions, a maximum daily exposure of 0.1-0.2 µg *N*-nitrosamine may occur. This amount does not represent a significant contribution to the total daily nitrosamine burden.

### Acknowledgements

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# OCURRENCE OF VOLATILE NITROSAMINES IN FOOD SAMPLES COLLECTED IN THREE HIGH-RISK AREAS FOR NASOPHARYNGEAL CARCINOMA

S. Poirier<sup>1</sup>, A. Hubert<sup>1</sup>, G. de-Thé<sup>1</sup>, H. Ohshima<sup>2</sup>, M.-C. Bourgade<sup>2</sup> & H. Bartsch<sup>2</sup>

<sup>1</sup>CNRS Laboratory of Epidemiology and Immunovirology of Tumours,  
Faculty of Medicine, A. Carrel, Lyon; and <sup>2</sup>International Agency  
for Research on Cancer, 150 cours Albert Thomas, Lyon, France

Fifty-four samples of foods consumed frequently in Tunisia, southern China and Greenland, all high-risk areas for nasopharyngeal carcinoma (NPC), were analysed for the presence of volatile nitrosamines by gas chromatography (GC) combined with a thermal energy analyser (TEA). Relatively high levels of *N*-nitrosodimethylamine (NDMA), *N*-nitrosopiperidine (NPIP) and *N*-nitrosopyrrolidine (NPYR) were found in Tunisian stewing base (*toukllia*) and dried mutton preserved in olive oil (*qaddid*). In one Chinese salted and dried fish sample, a high level of NDMA (133 µg/kg) was detected, but for the 14 others the levels ranged from undetectable to 14 µg/kg, with a mean of 3 µg/kg. Similarly high levels of NDMA were found in Eskimo dried, unsalted fish samples. NDMA, NPIP and NPYR at various levels were present in Chinese vegetables fermented in brine. The possible role of nitrosamines in the etiology of NPC is discussed.

NPC is prevalent in particular in three widely different populations — Chinese in south-east Asia, Arabs in North Africa and Eskimos in the Arctic (de-Thé, 1982). Etiological factors that have been associated with NPC include infection by Epstein-Barr virus and genetic and environmental factors. Several epidemiological studies have indicated that food habits could be associated with NPC (Geser *et al.*, 1978; Armstrong *et al.*, 1983; Li, C.C. *et al.*, 1985; Yu *et al.*, 1985, 1986). Since many volatile nitrosamines are known to induce tumours in the nasal cavity of experimental animals (Magee *et al.*, 1976), we have analysed for these nitrosamines in representative preserved foods collected in the three high-risk areas for NPC — Tunisia, southern China and Greenland (Hubert & de-Thé, 1986).

## Food samples and analysis of volatile nitrosamines

The most common types of foods (dried, salted and fermented) were collected from families or at local markets in various towns in Tunisia, Macao, Guangxi in China and on the west coast of Greenland. Of the 100 samples collected, 54 were selected for analysis of volatile nitrosamines in view of consumption frequency and type of preservation used: 40/54 of the samples tested are consumed more than three times a week and the rest once or twice a week.

Ten grams of homogenized sample were distilled *in vacuo* according to the method of Fine (1978). Volatile nitrosamines were analysed by GC-TEA. The occurrence of the compounds in the food extracts was further confirmed by high-performance liquid chromatography-TEA analysis (Fan *et al.*, 1978).

### Tunisian foods

As shown in Table 1, several nitrosamines were detected in three samples of Tunisian foods that are consumed daily. *Touk lia* (stewing base), which is the main ingredient of Tunisian cooking, contained NDMA, NPIP and NPYR at concentrations of 12, 43 and 5.8  $\mu\text{g}/\text{kg}$ , respectively. *Qaddid* (home-made dried mutton preserved in olive oil) contained 23  $\mu\text{g}/\text{kg}$  NDMA and 3.4  $\mu\text{g}/\text{kg}$  NPYR. Since both *touk lia* and *qaddid* contain red and black peppers, paprika, salt and other spices, the nitrosamines found in these foods may originate from amines such as piperidine and pyrrolidine from these spices, which might react with nitrite to form the nitrosamines (Sen *et al.*, 1973). NDMA was also detected in turnips fermented in brine at a concentration of 3.0  $\mu\text{g}/\text{kg}$ . Other Tunisian food samples, such as salted anchovies, *harissa* (spice mixture) and *louben* (sap from the mastic tree) contained only trace amounts or undetectable levels of volatile nitrosamines.

**Table 1. Occurrence of volatile nitrosamines in food samples collected in Tunisia**

Food sample	No. of samples analysed	Level of nitrosamine detected ( $\mu\text{g}/\text{kg}$ )
<i>Qaddid</i> (dried mutton preserved in olive oil)	1	NDMA (23), NPYR (3.4)
<i>Touk lia</i> (stewing base)	1	NDMA (12), NPIP (43), NPYR (5.8)
Turnips fermented in brine	1	NDMA (3.0)
<i>Harissa</i> (spice mixture)	1	NDMA (trace), NPYR (trace)
Other food samples <sup>a</sup>	6	None

<sup>a</sup>Salted anchovies, mixed vegetables fermented in brine, olives fermented in brine, *louben* (sap from the mastic tree), olive oil and *sman* (fermented and preserved butter)

### Chinese foods

Table 2 shows the levels of volatile nitrosamines in Chinese foods. In one of the 15 fish samples analysed, the level of NDMA was 133  $\mu\text{g}/\text{kg}$ , but for the 14 others levels ranged from undetectable to 14  $\mu\text{g}/\text{kg}$ , with a mean of 3  $\mu\text{g}/\text{kg}$ . Fish in southern China is prepared by salting in brine and drying under the sun ('tough' or 'hard meat' salted fish). Sometimes the fish is allowed to soften by decomposition before salting, to prepare 'soft meat' salted fish (Yu *et al.*, 1986). Variations in the levels of NDMA may reflect different modes of preparation or even individual differences within a given way of preparation. The levels of NDMA in Chinese fish samples detected in the present study are consistent with those reported by Huang *et al.* (1977, 1978a, 1981) and Tannenbaum *et al.* (1985).

**Table 2. Occurrence of volatile nitrosamines in food samples collected in southern China**

Food sample	No. of samples analysed	Level of nitrosamines detected ( $\mu\text{g}/\text{kg}$ )
<i>Hard, salted, dried fish</i>		
Grouper ( <i>Epinephelus asciticus</i> )	1	NDMA (133)
Croaker ( <i>Pseudosciaena</i> )	4	NDMA 1.0; 0.3; 2.0)
Japanese mackerel ( <i>Scomber japonicus</i> )	1	NDMA (1.1)
Spotted mackerel ( <i>Scomber iapinocephalus</i> )	1	NDMA (1.0)
Silver pomfret ( <i>Pampus argenteus</i> )	1	NDMA (0.2)
Dolphin ( <i>Coryphaena hippurus</i> )	1	NDMA (9.2)
Soldier croaker ( <i>Pseudosciaena anea</i> )	1	NDMA (0.3)
Ribbon fish ( <i>Trichiumus haumela</i> )	1	NDMA (6.4)
Sprats small fry ( <i>Dussumieria acuta</i> )	1	NDMA (1.5)
Ribbon small fry ( <i>Trichiumus haumela</i> )	1	NDMA (14)
Croaker small fry ( <i>Pseudosciaena anea</i> )	1	NDMA (1.0)
<i>Soft, salted, dried fish</i>		
Japanese mackerel ( <i>Scomber japonicus</i> )	1	NDMA (4)
<i>Lap cheung</i> sausage	1	NDMA (1.2)
<i>Lap yok</i> salted pork	1	NDMA (0.5)
Green mustard leaves ( <i>Brassica juncea</i> ) fermented in brine	1	NDMA (13), NPIP (14), NPYR (18)
Radish roots and stem fermented in brine	2	NDMA (4.3; 6.0), NPYR (2.4)
Chinese cabbage fermented in brine	3	NDMA (2.7; 0.6; 6.1), NPIP (trace 9.2), NPYR (13; 5.5; 96)
Fermented soya bean paste	3	NPYR (5.8)
Other foods <sup>a</sup>	6	None

<sup>a</sup>Fermented shrimp-fish paste, *tao-si* soya beans, salted olives, plums fermented in brine, dried 'dates' (*Zizyphus jujuba* Mill) and dried persimmon (*Diospyros kaki*)

Significant levels of NDMA, NPIP and NPYR were observed in Chinese pickled vegetables, such as green mustard leaves, Chinese cabbage, radish roots and stem fermented in brine, with highest values of 13, 14 and 96  $\mu\text{g}/\text{kg}$ , respectively. *Lap cheung* (Chinese sausage) and *lap yok* (salted pork) contained NDMA at levels of 1.2 and 0.5  $\mu\text{g}/\text{kg}$ , respectively. Other foods, such as *tao-si* (soya beans), dried 'dates' and persimmon contained no detectable level of volatile nitrosamines.

### Greenland foods

The four fish samples from Greenland tested were all found to contain NDMA, at levels ranging from 8.6 to 38  $\mu\text{g}/\text{kg}$  (Table 3). It should be noted that the mode of preparation of fish is different from that in China: in Greenland, dried fish is never salted. It is therefore

unlikely that the proposed mechanism for formation of nitrosamines in Cantonese-style fish (from nitrites present in pickling mixtures) is relevant to Greenland dried fish. Other food samples, such as dried reindeer, dried seal meat and semi-dried seal blubber, contained no detectable level of volatile nitrosamines.

**Table 3. Occurrence of volatile nitrosamines in food samples collected in Greenland**

Food sample	No. of samples analysed	Level of nitrosamines detected ( $\mu\text{g}/\text{kg}$ )
<i>Mikialak</i> , dried atlantic cod ( <i>Gadus morhua</i> )	1	NDMA (8.6)
<i>Uruvaq</i> , dried polar cod ( <i>Boreagadus saida</i> )	1	NDMA (26)
<i>Aalissaqaq</i> , Atlantic cod and head ( <i>Gadus morhua</i> )	1	NDMA (29)
<i>Amassat</i> , dried capelin fish ( <i>Mallotes villosous</i> )	1	NDMA (38)
Raw fjord-seal liver ( <i>Pusah ispidia</i> )	1	NDMA (trace)
Others <sup>a</sup>	7	None

<sup>a</sup>*Tuttu*, dried reindeer (*Rangifer tarandus*), raw fjord-seal meat (*Pusah ispidia*), dried fjord-seal meat, raw fjord-seal blubber, semi-dried fjord-seal blubber, fjord-seal oil and berries preserved in seal oil (*Vaccinium uliginosum* and *Empetrum nigrum*)

### Possible role of volatile nitrosamines in the etiology of NPC

A recent epidemiological study in Hong Kong showed a significantly increased risk for NPC due to consumption of salted fish during childhood. The relative risk for developing NPC was 20 for individuals who consumed Cantonese-style salted fish between the ages of one and two years and 38 for those who consumed such items by the age of ten (Yu *et al.*, 1986).

In the present study, some of the salted fish commonly consumed in high-risk areas of NPC in southern China were shown to contain relatively high amounts of NDMA. Dried fish samples from Greenland also contained NDMA at high concentrations. In addition, NDMA, NPIP and NPYR were shown for the first time to be present in typical Tunisian foods, such as dried mutton and stewing base. Some vegetables fermented in brine, and collected in southern China and Tunisia, also contained volatile nitrosamines. In comparison with the levels reported for most western foods (including preserved and fresh fish preparations; National Research Council, 1981; Sen *et al.*, 1985), foods collected in the three high-risk areas for NPC contained higher levels of volatile nitrosamines. Thus, exposure to these compounds in populations at high-risk for NPC could be higher. However, although the Japanese consume salted and dried fish as well as vegetables

fermented in brine, containing volatile nitrosamines at levels comparable to those detected in the present study (Kawabata *et al.*, 1979), the incidence of NPC in Japan is very low, with an age-adjusted mortality rate of  $<0.4/100\ 000$  inhabitants (Waterhouse *et al.*, 1982). This may be related to the fact that fish products are not eaten frequently during weaning or childhood in Japan, or that the fish products consumed in China contain additional, as yet unknown carcinogens.

From the present report, it is difficult to conclude whether or not volatile nitrosamines are involved in the etiology of NPC, since exposure to nitrosamines formed endogenously has not been evaluated. Further analyses of the collected food items for the presence of mutagens, total nitroso compounds and substances that may reactivate Epstein-Barr virus are in progress. In addition, epidemiological case-control studies are under way in the three high-risk areas to correlate NPC with consumption of food contaminated with volatile nitrosamines.

#### **Acknowledgements**

The work reported in this article was undertaken during the tenure of a Fellowship awarded to S. Poirier by the Ligue Nationale Française contre le Cancer. The authors are grateful to Mrs E. Heseltine, Miss S. Antony and Mrs M.-B. D'Arcy for editorial and secretarial assistance.

# **TOBACCO AND BETEL-QUID CARCINOGENESIS**

## INVESTIGATIONS ON THE MOLECULAR DOSIMETRY OF TOBACCO-SPECIFIC *N*-NITROSAMINES

S.S. Hecht, S.G. Carmella, N. Trushin, P.G. Foiles,  
D. Lin, J.M. Rubin & F.-L. Chung

*Division of Chemical Carcinogenesis, Naylor Dana Institute  
for Disease Prevention, American Health Foundation, Valhalla, NY 10595, USA*

Approaches for assessing molecular dosimetry of 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK) and *N'*-nitrosornicotine (NNN) in humans by measurement of haemoglobin or DNA adducts are discussed. NNK and NNN form haemoglobin adducts in Fischer 344 rats. Acid or base hydrolysis of the globin gives 4-hydroxy-1-(3-pyridyl)-1-butanone, which can be detected in rat blood up to six weeks after injection of NNK; it may be a useful marker for assessing uptake and metabolic activation of NNK and NNN in tobacco consumers. NNK and its major metabolite, 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanol (NNAI), methylated DNA of rat liver, lung and nasal mucosa to similar extents. NNAI is formed in human tissues from NNK, but immunoassays for *O*<sup>6</sup>-methyldeoxyguanosine (*O*<sup>6</sup>-medGuo) in exfoliated oral cells from snuff-dippers have been negative. NNK is also expected to form pyridyloxobutyl adducts in DNA; <sup>32</sup>P-postlabelling assays for these adducts are being developed and appear to hold promise for detecting NNK- or NNN-DNA adducts *in vivo*.

According to the *IARC Monographs on the Evaluation of the Carcinogenicity of Chemicals to Humans*, 'The occurrence of malignant tumours of the respiratory tract and of the upper digestive tract is causally related to the smoking of different forms of tobacco; the occurrence of tumours of the bladder, renal pelvis and pancreas is causally related to the smoking of cigarettes.' In addition, an IARC working group concluded that oral use of smokeless tobacco is carcinogenic to humans (IARC, 1985b, 1986b). Tobacco-specific *N*-nitrosamines are considered to be among the most important carcinogens in tobacco smoke and are quantitatively the major carcinogens present in unburnt tobacco. Therefore, a growing consensus has evolved that they are causative agents in cancers associated with tobacco use (Craddock, 1983; Bartsch & Montesano, 1984; Hoffmann & Hecht, 1985). Although extensive studies have documented the concentrations of tobacco-specific *N*-nitrosamines in unburnt tobacco and in mainstream and sidestream tobacco smoke (Hoffmann & Hecht, 1985), little is known about individual uptake and metabolic activation of these carcinogens in humans. The main markers for tobacco and tobacco-smoke uptake by humans that have been used in studies to date are nicotine, cotinine, carboxyhaemoglobin and thiocyanate (IARC, 1986b). These studies have clearly shown that machine measurement of cigarette smoke components is not a reliable indicator of individual uptake.

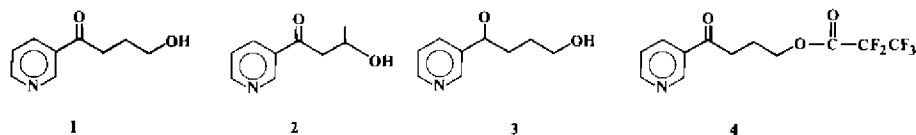


Many factors, such as extent of inhalation and exposure to sidestream smoke, can affect the dose of particular tobacco-smoke constituents. For *N*-nitrosamines, estimation of dose is further complicated by their endogenous formation, which has been demonstrated in several studies on smokers (Hoffmann & Brunnemann, 1983; Bartsch & Montesano, 1984; Ladd *et al.*, 1984b). Metabolic studies of tobacco-specific *N*-nitrosamines in cultured human tissues have shown wide interindividual variations in metabolic activation; this has also been observed for other carcinogens such as benzo[*a*]pyrene (Astrup *et al.*, 1980; Castonguay *et al.*, 1983a). In order to bypass these uncertainties in estimating dose, reliable measures of *N*-nitrosamine uptake and activation are needed. Such assays would be exceedingly useful in epidemiological studies on tobacco use and cancer development, to provide a quantitative index of carcinogen dose. Markers for uptake and activation of two tobacco-smoke carcinogens, benzo[*a*]pyrene and 4-aminobiphenyl, are being developed, involving measurement of DNA adducts and haemoglobin adducts (Perera *et al.*, 1982; Green *et al.*, 1984; Garner, 1985; Harris *et al.*, 1985). This paper reviews our current work related to haemoglobin binding and DNA binding of NNK and NNN, the two most carcinogenic tobacco-specific *N*-nitrosamines.

### Haemoglobin binding of NNK and NNN

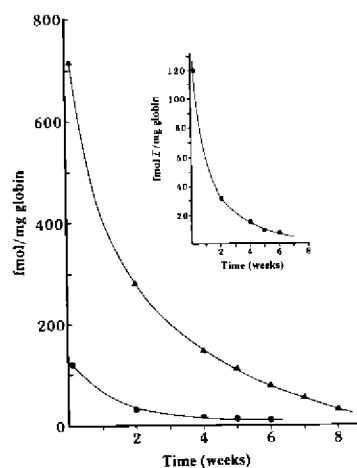
In preliminary studies, [ $^3\text{H}$ ]NNK (1 Ci/mmol, 0.1 mCi) was administered by subcutaneous injection to each of two male Fischer 344 rats. The rats were killed 24 h later, and globin was isolated as described by Green *et al.* (1984). Combustion analysis demonstrated the presence of bound tritium (0.04% of the dose). Approximately 10-15% of the bound radioactivity was found to be labile upon treatment of globin with aqueous 0.1 N sodium hydroxide for 1 h at room temperature or with aqueous 1 N hydrochloric acid for 2.5 h at room temperature. It was not released upon washing the globin with water or trichloromethane, or upon incubation with water for 16 h at 37°C, pH 4 or pH 7. The released radioactivity was extracted with trichloromethane and analysed by reverse-phase and normal-phase high-performance liquid chromatography (HPLC). In both systems, the majority of the radioactivity coeluted with 4-hydroxy-1-(3-pyridyl)-1-butanone (**1**, Scheme 1). Radioactivity also coeluted with **1** upon analysis by thin-layer chromatography, under conditions that separated **1** from the isomeric keto alcohol **2**. Treatment of the radioactive material with sodium borohydride gave a product identical in HPLC retention time to the diol **3**. Derivatization of the radioactive material with pentafluoropropionic anhydride gave a product identical in retention time to the pentafluoropropionate **4**. All of these results are consistent with **1** being the structure of the compound released upon base or acid treatment of globin from NNK-treated rats. To confirm this, ten rats were each given daily intraperitoneal injections of 100 mg/kg NNK for three days and killed 24 h later. The globin was isolated, and a 900-mg aliquot was treated with base. The resulting extract was purified by HPLC, silylated and analysed by capillary gas chromatography-mass spectrometry. The mass spectrum of the trimethylsilyl derivative of **1** was identical to that of a standard.

Scheme 1



Since the keto alcohol *l* is a metabolite of NNK, it seemed possible that the globin may have been contaminated with trace amounts of unbound *l*. In order to assess this possibility and to determine the persistence of the precursor to *l* in globin, two rats were each given an intraperitoneal injection of [ $5\text{-}^3\text{H}$ ]NNK (1 Ci/mmol, 2 mCi); globin was isolated at intervals from 24 h to eight weeks. Total binding was determined by counting an aliquot of the globin, and release of *l* upon base treatment was assessed by HPLC. The results, illustrated in Figure 1, show that *l* was detectable throughout a six-week period. This demonstrates that *l* was formed from a globin adduct, since unbound *l*, formed metabolically from NNK, is cleared from blood in less than 24 h. However, the globin adduct that yields *l* upon hydrolysis was less stable ( $t_{1/2} \approx 9$  days) than globin itself ( $t_{1/2} \approx 29$  days; Port & Thurnam, 1983).

**Fig. 1.** Levels of total globin adducts ( $\blacktriangle$ ) and of *l* ( $\bullet$ ; see Scheme 1) released upon base treatment of globin at intervals after treatment of two rats with [ $5\text{-}^3\text{H}$ ]NNK by intraperitoneal injection



Points are the means of values from the two rats. The 24-h point corresponds to approximately 0.1% of dose for total adducts, and 0.01% for *l*, on the basis of blood volume being equal to 6.4% of a rat's body weight (Ringler *et al.*, 1979).

ate DNA *in vivo* (Hecht *et al.*, 1980b). This has been observed in several recent studies (Castonguay *et al.*, 1984; Chung *et al.*, 1985; Foiles *et al.*, 1985; Belinsky *et al.*, 1986a; Hecht *et al.*, 1986a). The promutagenic adducts *O*<sup>6</sup>-methylguanine (*O*<sup>6</sup>-meG) and *O*<sup>4</sup>-methylthymidine, as well as 7-methylguanine (7-meG), have been detected in the target tissues, nasal mucosa, liver and lung, after acute or chronic treatment of rats with NNK. *O*<sup>6</sup>-meG persists in lung DNA after chronic treatment. These events appear to be related to tumour induction by NNK. However, little is known about the mechanism of DNA methylation by

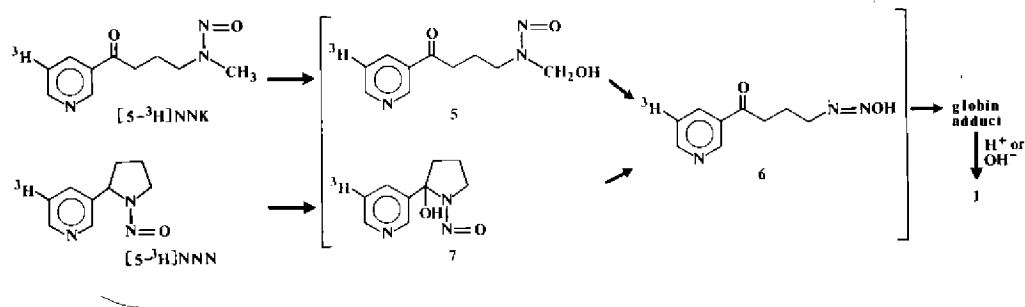
The structure of the globin adduct precursor to *l* is not known. Metabolic activation of NNK by methyl hydroxylation to 5 (Scheme 2) should give the diazohydroxide  $\delta$ . This could react with one of the nucleophilic sites in globin to give an adduct that is labile to acid or base hydrolysis. The structure of this adduct is under investigation. If the adduct were formed *via* the diazohydroxide  $\delta$ , it should also be detected in rats treated with NNN. To test this hypothesis, two Fischer 344 rats were each given an intraperitoneal injection of [ $5\text{-}^3\text{H}$ ]NNN (1 Ci/mmol, 2 mCi) and were killed at intervals as described above. Treatment of the globin with base and analysis by HPLC gave a single radioactive peak with a retention time identical to that of *l*. The amount of *l* formed was 19 fmol/mg globin after 24 h — about 16% of the amount detected from [ $5\text{-}^3\text{H}$ ]NNK. The total binding of [ $5\text{-}^3\text{H}$ ]NNN to globin was approximately 0.05% after 24 h — about 50% of the [ $5\text{-}^3\text{H}$ ]NNK level. These results support the hypothesis that  $\delta$  yields the globin adduct. However, further studies are required to identify *l* positively, as formed from [ $5\text{-}^3\text{H}$ ]NNN.

Since sensitive methods for detection of *l* can be developed, these results provide a good lead for using haemoglobin as a dosimeter for exposure to NNK and NNN.

### DNA binding of NNK

Hydroxylation of the methylene group  $\alpha$  to the *N*-nitroso functionality of NNK occurs metabolically, yielding methyldiazohydroxide or a related methylating agent. Therefore, NNK was expected to methylate DNA *in vivo* (Hecht *et al.*, 1980b). This has been observed in several recent studies (Castonguay *et al.*, 1984; Chung *et al.*, 1985; Foiles *et al.*, 1985; Belinsky *et al.*, 1986a; Hecht *et al.*, 1986a). The promutagenic adducts *O*<sup>6</sup>-methylguanine (*O*<sup>6</sup>-meG) and *O*<sup>4</sup>-methylthymidine, as well as 7-methylguanine (7-meG), have been detected in the target tissues, nasal mucosa, liver and lung, after acute or chronic treatment of rats with NNK. *O*<sup>6</sup>-meG persists in lung DNA after chronic treatment. These events appear to be related to tumour induction by NNK. However, little is known about the mechanism of DNA methylation by

Scheme 2

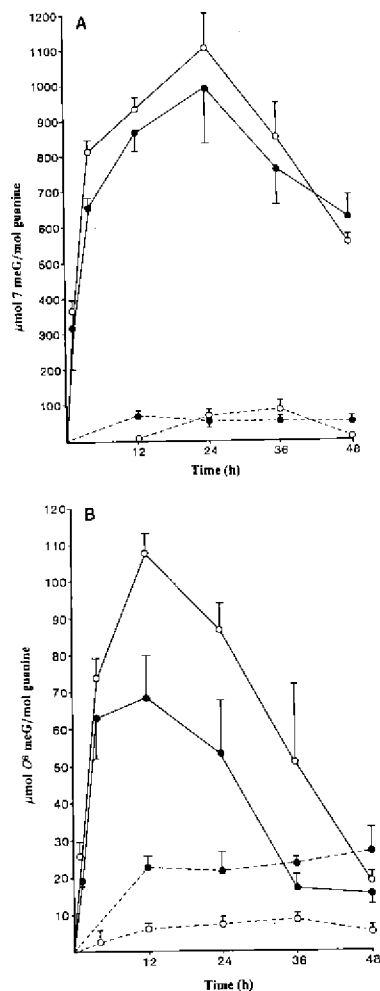


NNK or its metabolites. Such information would be valuable for interpreting the results of experiments in which methylated DNA might be detected in tobacco consumers. NNK is rapidly metabolized in experimental animals and in cultured human tissues to the alcohol, NNAI (Castonguay *et al.*, 1983a,b; Adams *et al.*, 1985). In order to explore the possible role of NNAI in DNA methylation by NNK, we carried out a comparative study. Groups of Fischer 344 rats were treated by subcutaneous injection with 0.39 mmol/kg NNAI or NNK. 7-meG and *O*<sup>6</sup>-meG were measured in DNA of liver, lung, and nasal mucosa by HPLC with fluorescence detection. The results for liver and lung DNA are presented in Figure 2. The levels of 7-meG and *O*<sup>6</sup>-meG formed from NNK and NNAI were similar.

In nasal mucosa, levels of 7-meG formed from NNK and NNAI were similar, but *O*<sup>6</sup>-meG formation from NNK exceeded that from NNAI (data not shown). These results indicate that racemic NNAI is a methylating agent; therefore, NNAI is probably not a detoxification product of NNK in rats. Since NNK and NNAI can be interconverted *in vivo*, it is not known whether or not the ultimate methylating agent is derived from NNK or NNAI. Some insight into this question might be obtained by investigating the *in-vivo* formation of the NNAI enantiomers and comparing their methylating activities. These studies are in progress.

Since NNK methylates DNA relatively efficiently at low doses (Belinsky *et al.*, 1986b), it may be possible to use DNA methylation as a dosimeter for human activation of tobacco-associated nitrosamines. We have investigated the application of the immuno-slot-blot method (Nehls *et al.*, 1984) to this problem. We used this assay to study levels of *O*<sup>6</sup>-medGuo in DNA from exfoliated oral cavity cells of smokeless tobacco users. The samples were obtained with the cooperation of Colonel George Palladino of a nearby military academy and were collected by scraping the inside of the mouth with a toothbrush. The mouth and toothbrush were rinsed with saline and the samples stored on ice. Within 4 h of collection, the saline solution was centrifuged at  $500 \times g$  for 5 min, the supernatant discarded, and the cell pellet frozen. DNA was purified from the frozen cell pellets using chloroform-isoamyl alcohol extraction and elution from hydroxylapatite. The DNA was made single-stranded by boiling, and 5  $\mu\text{g}$  were applied by vacuum filtration to a nitrocellulose membrane and dried for 2 h at 80°C. After blocking the membrane with a solution of 1% bovine serum albumin and 0.5% gelatin overnight, the membrane was treated sequentially with affinity-purified rabbit anti-*O*<sup>6</sup>-medGuo antibody (Foiles *et al.*, 1985), and alkaline phosphatase-

**Fig. 2.** Levels of 7-meG (A) and *O*<sup>6</sup>-meG (B) in DNA of liver (—) and lung (---) at intervals after subcutaneous injection of 0.39 mmol/kg of NNK (○) or NNAL (●)



not more than 0.1  $\mu\text{mol/mol}$  deoxyguanosine coeluted with 9; the identity of this peak requires further investigation. These results demonstrated that the levels of 9 formed from NNK in rats are low, if 9 is formed at all. Therefore, we have investigated the <sup>32</sup>P-postlabelling assay, as originally described by Gupta *et al.* (1982), for analysis of 9 and related NNK adducts. We have used 9 as a model for these initial studies because it is the only characterized deoxyribonucleoside related to NNK and NNN that is currently available. The <sup>32</sup>P-postlabelling assay, if successful, would be applicable to human samples.

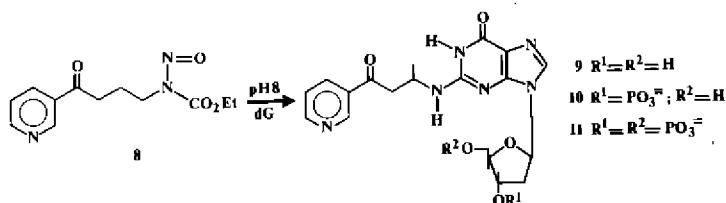
labelled goat anti-rabbit immunoglobulin G antibody. The membrane was then treated with the substrate solution containing 5-bromo-4-chloro-3-indolylphosphate and nitro-blue tetrazolium. This produces a purple spot on the membrane in the presence of alkaline phosphatase. Using calf thymus DNA modified with a known level of *O*<sup>6</sup>-med-Guo, this assay could detect 5  $\mu\text{mol}$  *O*<sup>6</sup>-med-Guo per mol deoxyguanosine, starting with 5  $\mu\text{g}$  DNA.

DNA from five snuff users and one nonuser was assayed; we were unable to detect *O*<sup>6</sup>medGuo in any of these samples. These results suggest that methylation, if it occurs, does so at levels below 5  $\mu\text{mol}$  *O*<sup>6</sup>-medGuo per mol deoxyguanosine. The sensitivity of our assay might be improved with the use of monoclonal antibodies. It is also possible that exfoliated cells are not the most appropriate cell type to assay. Cells deeper in the mucosal layer may be more heavily modified.

In contrast to methylation, modification of DNA by the pyridyloxobutyl functionality of NNK should yield adducts that are characteristic of exposure to tobacco-specific nitrosamines. We have carried out model studies with 4-(carbethoxynitrosamino)-1-(3-pyridyl)-1-butanone (8, Scheme 3) which, upon hydrolysis, should yield the pyridyloxobutyl diazohydroxide 6 (Scheme 2). We established that 8 reacts with deoxyguanosine at pH 8 to give the *N*<sup>2</sup>-substituted derivative 9 (Scheme 3; Hecht *et al.*, 1986b). The reaction is less favoured at pH 7, and the product distribution is more complex.

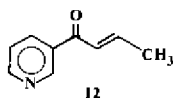
To examine the possible formation of 9 from NNK *in vivo*, Fischer 344 rats were treated by intraperitoneal injection on three consecutive days with [5-<sup>3</sup>H]NNK (total dose, 5 mCi/5  $\mu\text{mol}$  per rat). The DNA was isolated from liver, lung and nasal mucosa, hydrolysed enzymatically to deoxyribonucleosides and analysed by reverse-phase HPLC. In the liver DNA samples, a peak corresponding to

## Scheme 3



For development of the  $^{32}P$ -postlabelling assay, the 3'-monophosphate *10* (Scheme 3) and 3',5'-diphosphate *11* (Scheme 3) were required. The diastereomeric 3'-monophosphates *10* were prepared by reaction of deoxyguanosine-3'-monophosphate with the  $\alpha,\beta$ -unsaturated ketone *12* (Scheme 4). In previous studies, we have shown that *12* reacts with deoxyguanosine to give *9*, as a mixture of diastereomers (Hecht *et al.*, 1986b). The 3'-monophosphates *10* were formed as a pair of diastereomers which were isolated by HPLC and had ultra-violet spectra similar to those of *9*. Treatment of *10* with nuclease  $P_1$ , a 3'-phosphatase, gave *9*. Treatment of *10* with T4 polynucleotide kinase gave a pair of diastereomeric 3',5'-diphosphates *11*. Treatment of the diphosphates with nuclease  $P_1$  and alkaline phosphatase again regenerated *9*. These results are consistent with the structures of *10* and *11*.

## Scheme 4



For the analysis of *9*, the DNA from NNK- or NNN-treated animals is degraded enzymatically to 3'-monophosphates. The 3'-monophosphates are separated from unmodified 3'-monophosphates by reverse-phase HPLC. This simplifies the  $^{32}P$ -postlabelling procedure since the bulk of the material, which consists of unmodified 3'-

monophosphates, is separated prior to labelling. A three-dimensional reverse-phase thin-layer chromatography system was developed to separate the diphosphate *11* from ATP and other material in the sample. This system was used for labelling *10* with  $\gamma$ - $^{32}P$ -ATP, catalysed by T4 polynucleotide kinase. The results of this analysis showed a single radioactive spot, which coeluted with standard *11*, as detected by its ultra-violet absorption. These results demonstrate the feasibility of using  $^{32}P$ -postlabelling for adducts such as *9*. Experiments using DNA from NNK-treated animals are in progress.

## Conclusions

Experiments carried out to date have demonstrated that both NNK and NNN form haemoglobin adducts, and that the keto alcohol *1*, formed upon hydrolysis of one of the adducts, may be a useful monitor for uptake and activation of these *N*-nitrosamines. This approach appears to be promising for studies on tobacco consumers, and work in this direction is in progress. A major advantage of this method, as noted by others, is the relative ease of obtaining sufficient material for analysis. A disadvantage is that the relationship of such adducts to carcinogenesis is more uncertain than that of DNA adducts. The studies in progress with the  $^{32}P$ -postlabelling technique, as applied to NNK and NNN, may provide

information on DNA modification by NNK and NNN in humans. It will be important to carry out studies with experimental animals during chronic dosing of NNK and NNN under conditions known to produce tumours. Measurement of haemoglobin adduct levels and DNA adduct levels in target tissues under these chronic dosing conditions will provide information on the extent to which haemoglobin adducts might reflect critical damage to DNA in target tissues.

**Acknowledgements**

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**MOLECULAR DOSIMETRY OF O<sup>6</sup>-METHYLGUANINE  
FORMATION AND CELL TOXICITY  
IN LUNG AND NASAL MUCOSA BY 4-(N-NITROSO-  
METHYLAMINO)-1-(3-PYRIDYL)-1-BUTANONE**

S.A. Belinsky<sup>1,3</sup>, J.A. Swenberg<sup>2</sup> & M.W. Anderson<sup>1</sup>

<sup>1</sup>Laboratory of Biochemical Risk Analysis, National Institute  
of Environmental Health Sciences, Research Triangle Park, NC 27709;  
and <sup>2</sup>Department of Biochemical Toxicology and Pathology,  
Chemical Industry Institute of Toxicology, Research Triangle Park,  
NC 27709, USA

The molecular dosimetry of O<sup>6</sup>-methylguanine (O<sup>6</sup>-meG) in DNA from lung and nasal mucosa was determined during administration of 4-(N-nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK) to Fischer 344 rats. O<sup>6</sup>-MeG accumulated in lung during 12 days of treatment with doses of NNK ranging from 0.1 to 100 mg/kg per day. The dose-response to NNK was nonlinear; the ratio of O<sup>6</sup>-meG to dose, an index of alkylation efficiency, increased dramatically as the dose of carcinogen decreased. These data suggest that high and low  $K_m$  pathways may exist for activation of NNK to a methylating agent. Clara cells, when compared to Type-II cells, macrophages and alveolar small cells, were found to possess the greatest concentration of O<sup>6</sup>-meG. Moreover, as the dose of NNK was decreased, a marked increase in the alkylation efficiency of Clara cells was observed. Thus, the presence of a high-affinity, low- $K_m$  pathway in Clara cells for activation of NNK may be a significant factor in the carcinogenicity of this tobacco-specific carcinogen.

The dose-response for O<sup>6</sup>-meG differed considerably between respiratory and olfactory mucosa. The dose-response to NNK was nonlinear in respiratory mucosa and linear in the olfactory mucosa, and the concentration of O<sup>6</sup>-meG was five times greater in respiratory than in olfactory mucosa after treatment with 1 mg/kg NNK. As the dose of NNK was increased, alkylation in the two regions of the nose became similar. Histological examination of the nasal passages following treatment with NNK indicated that the olfactory region was more sensitive than the respiratory region to toxicity induced by NNK. Since the majority of nasal tumours induced by NNK appear to originate in the olfactory region, these data indicate that both the formation of promutagenic adducts and cell proliferation secondary to toxicity are required for the initiation of neoplasia within the nose.

Previous studies (Belinsky *et al.*, 1986a) have demonstrated that multiple administrations of the tobacco-specific carcinogen NNK result in accumulation of the promutagenic adduct O<sup>6</sup>-meG in lung and nose and in toxicity in the nasal mucosa. The purpose of this study was to determine the molecular dosimetry of O<sup>6</sup>-meG in DNA from lung and nasal mucosa and the dose-response for toxicity in respiratory and olfactory mucosa during treatment with NNK.

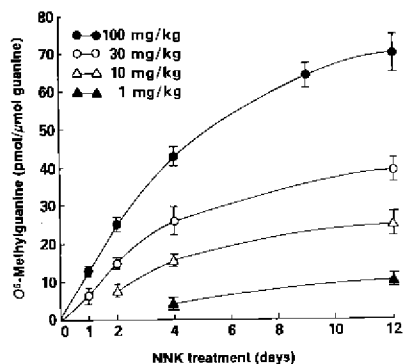
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<sup>3</sup>To whom correspondence should be addressed

### Low-dose accumulation of $O^6$ -meG in lung

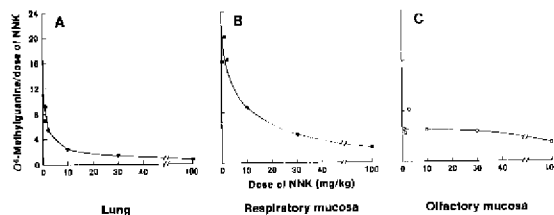
The concentration of  $O^6$ meG in DNA from lungs of Fischer 344 rats was determined over 12 days of exposure to NNK.  $O^6$ -MeG accumulated with doses of NNK ranging from 0.1 to 100 mg/kg per day (Fig. 1; Belinsky *et al.*, 1986c). A plot of adduct levels as a function of dose after 12 days of treatment revealed that the dose-response for  $O^6$ -meG accumulation was nonlinear (Belinsky *et al.*, 1986c). The amount of  $O^6$ -meG formed per unit dose of NNK, an index of efficiency of alkylation, increased dramatically as the dose of NNK decreased (Fig. 2A). The metabolism of NNK to a methylating agent is thought to occur by cytochrome P450-dependent  $\alpha$ -hydroxylation (Hecht *et al.*, 1980b). Recently, several different cytochrome P450 isozymes have been identified in rat lung (Domin *et al.*, 1984). On the basis of the two-component nature of the curve (Fig. 2A), we hypothesize that low- and high- $K_m$  pathways exist for activation of NNK to a methylating agent in the lung.

**Fig. 1. Accumulation of  $O^6$ -meG in lung following treatment with NNK**



Male Fischer 344 rats (175-200 g) were treated intraperitoneally for 12 consecutive days with 0.1, 0.3, 1, 10, 30 or 100 mg/kg NNK and killed 4 h after treatment on days 1, 2, 4 and 12. DNA was isolated from lung by digestion with pronase followed by phenol-chloroform extraction and ethanol precipitation (Belinsky *et al.*, 1986a). The concentrations of  $O^6$ -meG in lungs of rats treated with 10, 30 or 100 mg/kg NNK were determined by fluorescence-linked high-performance liquid chromatography, as described elsewhere (Belinsky *et al.*, 1986a). Limits of detection were 1.5 pmol/ $\mu$ mol unmodified base.  $O^6$ -meG in lungs from rats treated with 1 mg/kg was determined by competitive radioimmune assay (Wild *et al.*, 1983; Belinsky *et al.*, 1986c). The antibody to  $O^6$ -methyldeoxyguanosine was a gift from Dr Roy Saffhill. Limits of detection were 0.2 pmol/ $\mu$ mol unmodified base. Values are means  $\pm$  SE from four rats.

**Fig. 2. Efficiency of  $O^6$ -meG formation**



The concentrations of  $O^6$ meG (pmol/ $\mu$ mol guanine) in lungs (A) after 12 days of treatment with NNK was divided by the dose of carcinogen (mg/kg per day) and plotted against dose. The concentration of  $O^6$ -meG in respiratory mucosa (B) and olfactory mucosa (C) after one day of treatment with NNK was divided by the dose of carcinogen and plotted against dose.

The accumulation of  $O^6$ -meG was found to correlate inversely with the depletion of  $O^6$ -meG DNA methyltransferase activity in lungs from rats treated for 12 days with doses of NNK ranging from 10 to 100 mg/kg per day (Belinsky *et al.*, 1986c). Thus, the accumulation of  $O^6$ -meG in lungs during treatment with high doses of carcinogen probably results in part from this depletion. However, in the dose range associated with the most rapid accumulation of  $O^6$ -meG (0.1-1.0 mg/kg), there was no apparent effect on repair activity in whole lung.

### Cell-specific differences in $O^6$ -meG formation

Experiments were carried out to determine the concentration of  $O^6$ -meG in Clara cells, Type-II cells, macrophage and alveolar small cells (predominantly endothelial cells) following treatment



of rats for four days with 0.3 or 100 mg/kg NNK. Alkylation in Clara cells was significantly greater than in all other lung-cell types following treatment with 0.3 mg/kg NNK (Clara cell > macrophage > alveolar small cell > Type-II cell; 28.2, 3.8, 1.5 and 1.1 pmol/ $\mu$ mol guanine, respectively). The greater extent of alkylation observed in the Clara cell is consistent with the reported localization of cytochrome P450 monooxygenase activity within this cell population (Jones *et al.*, 1983). The distribution of *O*<sup>6</sup>-meG in lung cells following treatment with a high dose of NNK (100 mg/kg per day) differed considerably from that following low-dose exposure. No more than a six-fold difference in adduct formation was observed among pulmonary cell types from rats treated with high doses of NNK (Belinsky *et al.*, 1986c). The dose-dependent difference in alkylation efficiency among pulmonary cell types was best demonstrated by the efficiency of *O*<sup>6</sup>-meG formation, which was found to increase 38-fold in Clara cells and only eight- to ten-fold in the other lung cell populations as the dose of NNK was decreased from 100 to 0.3 mg/kg (Belinsky *et al.*, 1986c). Thus, the localization of a high-affinity pathway for the activation of NNK in Clara cells may be an important factor in the strong carcinogenicity of this compound in rodent lung.

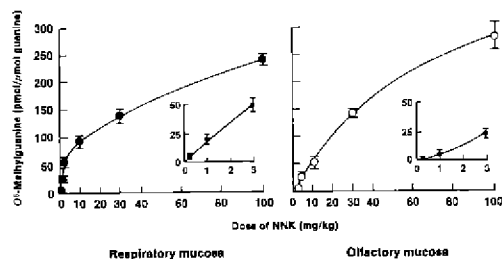
#### Dose-response for cytotoxicity and *O*<sup>6</sup>-meG in the nose

Histopathological examination of the nasal passages revealed dose-related, cell-specific differences in toxicity following treatment of rats with 1, 10, 30 or 100 mg/kg NNK for 12 days. No toxicity was observed in the nose when 1 mg/kg NNK was administered. Bowman's glands and Steno's gland were the most sensitive site for toxicity, exhibiting necrosis after as little as two days of treatment with 10 mg/kg NNK (Belinsky *et al.*, 1986d). Damage to these glands progressed in a dose- and time-dependent manner. Serous glands present in the respiratory region were resistant to the toxicity of NNK. Respiratory epithelium exhibited only mild cell toxicity when NNK (100 mg/kg) was administered for four to 12 days. These data indicate that the olfactory region of the nose is more sensitive to toxicity induced by NNK than is the respiratory region.

The molecular dosimetry of *O*<sup>6</sup>-meG formation differed considerably between respiratory and olfactory mucosa. The relationship between dose and *O*<sup>6</sup>-meG formation was nonlinear in respiratory mucosa after one day of treatment with NNK. The slope of the line was very steep with doses of NNK ranging from 0.3 to 3.0 mg/kg but became markedly less steep in the dose range 10-100 mg/kg (Fig. 3). Thus, as observed in the lung, the efficiency of alkylation increased in respiratory mucosa as the dose of NNK was decreased (Fig. 2B). In contrast, the dose-response in the olfactory mucosa appeared to be linear (Fig. 3), and the alkylation efficiency in this region of the nose did not change as the dose of NNK was altered (Fig. 2C).

Differences in the concentration of *O*<sup>6</sup>-meG in respiratory and olfactory mucosa were also observed over the dose range of NNK employed in this study. The concentration of *O*<sup>6</sup>-meG was five times greater in respiratory than olfactory mucosa after treatment with 1 mg/kg NNK (Fig. 3). As the dose of NNK was increased, differences in the formation of *O*<sup>6</sup>-meG between the two regions of the nose decreased, such that the concentration of this adduct was similar in both regions after treatment with either 30 or 100 mg/kg NNK. These data suggest that differences in the dose-response for *O*<sup>6</sup>-meG in the nose may result from localization of the low- $K_m$  pathway for NNK activation within the respiratory mucosa.

**Fig. 3. Dose-response for  $O^6$ -meG formation in nasal mucosa**



$O^6$ -MeG was determined in respiratory and olfactory mucosa from rats after one day of treatment with 0.3, 1.0, 3.0, 10, 30 or 100 mg/kg NNK. Experimental conditions were as described in the legend to Figure 1. Values are means  $\pm$  SE from three to five rats.

NNK originate in the olfactory region. Although formation of the promutagenic adduct  $O^6$ -meG was greater in respiratory mucosa than in olfactory mucosa after low doses of NNK, adduct concentrations were similar in both regions of the nose in the dose range that induced nasal tumours. However, these high doses of NNK were associated with marked cytotoxicity only in the olfactory region. Taken together, these data indicate that both the formation of promutagenic adducts and cell proliferation secondary to toxicity are required for the induction of neoplasia within the nose.

#### Acknowledgements

S.A. Belinsky was the recipient of a National Research Service Award (ESO5292) from the National Institute of Environmental Health Sciences.

#### Relationship between DNA alkylation, cytotoxicity and tumour formation in the nose

Bioassay results (Hoffmann *et al.*, 1984c) have demonstrated a sharp dose-response curve for the induction of tumours in the nasal passages of rats treated with NNK. The incidence of malignancy in the nose was approximately 55% in rats treated for 20 weeks with either 16 or 50 mg/kg NNK (three times/week); however, when the dose of NNK was decreased to 5.4 mg/kg, the incidence of malignant tumours was only 4%. Preliminary data from our laboratory suggest that the majority of malignant nasal tumours induced by

# LOCALIZATION OF BOUND METABOLITES IN THE RESPIRATORY TISSUES AND OESOPHAGUS BY HIGH-RESOLUTION MICROAUTORADIOGRAPHY IN FISCHER 344 RATS TREATED WITH *N*-NITROSONORNICOTINE

H. Tjälve<sup>1,3</sup> & A. Castonguay<sup>2</sup>

<sup>1</sup>Department of Pharmacology and Toxicology, The Swedish University of Agricultural Sciences, Uppsala Biomedical Centre, Box 573, S-751 23 Uppsala, Sweden; and <sup>2</sup>School of Pharmacy, Laval University, Quebec City, PQ, Canada G1K 7P4

High-resolution microautoradiography for detection of bound radioactivity in Fischer 344 rats given a single subcutaneous dose of <sup>3</sup>H-*N*-nitrosonornicotine (<sup>3</sup>H-NNN) showed a labelling of the olfactory region of the nose, which was restricted to the secretory cells of the glands of the lamina propria mucosae (Bowman's glands). In the oesophagus, bound radioactivity was present in the squamous epithelium, most marked in the middle and superficial cell layers. The olfactory nasal mucosa and the oesophagus are targets for the carcinogenicity of NNN in Fischer 344 rats. Our results indicate that the labelled cells in these tissues are the principal sites in which NNN is activated to alkylating species, and these cells may also be the ones that undergo malignant transformation. Bound radioactivity was also found in the lung (mainly in the Clara cells of the bronchi and bronchioles) and in the trachea (preferentially in the mucous cells), although NNN does not induce tumours in the lung or trachea of the rats. Why covalent binding of metabolites to cellular macromolecules is not correlated to carcinogenesis at these sites is not known.

The tobacco-specific *N*-nitrosamine NNN induces tumours in the oesophagus and in the nasal olfactory region in Fischer 344 rats when given in the drinking-water (Hoffmann *et al.*, 1975). When administered by subcutaneous injection, the olfactory region of the nose is the only site in which malignant tumours develop (Hecht *et al.*, 1980). Whole-body autoradiography of Fischer 344 rats treated with [<sup>2-14</sup>C]-NNN has shown a localization of radioactivity in the nasal olfactory mucosa and in the oesophagus (Brittebo & Tjälve, 1981). In the present study, high-resolution microautoradiography was used to examine the sites of binding of radioactivity in these tissues of Fischer 344 rats given <sup>3</sup>H-NNN. Since an accumulation of bound NNN metabolites will also occur in the lung and the trachea (Brittebo & Tjälve, 1981), these tissues were also processed for high-resolution microautoradiography.

## High-resolution microautoradiographic localization of bound metabolites

Two male Fischer 344 rats (~ 60 g) were each given a single subcutaneous injection of <sup>3</sup>H-NNN, labelled at carbon 5 in the pyrrolidine ring (the specific radioactivity was 200 mCi/mmol; the injected dose was 0.5 mCi/animal, corresponding to 7.4 mg/kg body weight). Four hours later, the animals were killed, and pieces of the olfactory portion of the nose, the oesophagus, the trachea and the lung were taken for high-resolution microscopic

<sup>3</sup>To whom correspondence should be addressed

autoradiography of bound radioactivity. In this procedure, the animals were anaesthetized with sodium pentobarbital and fixed *in situ* by vascular perfusion with 2.5% cacodylate-buffered glutaraldehyde (pH 7.4). Excised tissues were further fixed in the same fixative, postfixed in 2% cacodylate-buffered osmium tetroxide, dehydrated in an ethanol series and embedded in Agar 100 resin (Agar Aids, Essex, UK). Semi-thin sections (1  $\mu$ m) were cut on glass slides in an LKB ultramicrotome, and then coated in the dark with Kodak NTB-2 emulsion by dipping. Autoradiograms were exposed at +4°C for about three months and then developed, fixed and stained with toluidine blue. Since NNN and its unbound metabolites are soluble in aqueous media or ethanol, the autoradiographic procedure leaves only cell-bound radioactivity in the tissues.

In autoradiograms of the olfactory region of the nose, there was a strong labelling of the secretory cells of the glands of the lamina propria mucosae (Bowman's glands) (Fig. 1A). Silver grains were present both over the nuclei and the cytoplasm of the cells. The secretory granules, present towards the lumina of the acini, usually showed lower labelling than the other parts of the cells. Very few silver grains were observed over the olfactory epithelium or over structures such as blood vessels, connective tissues and fasciculi of olfactory nerves.

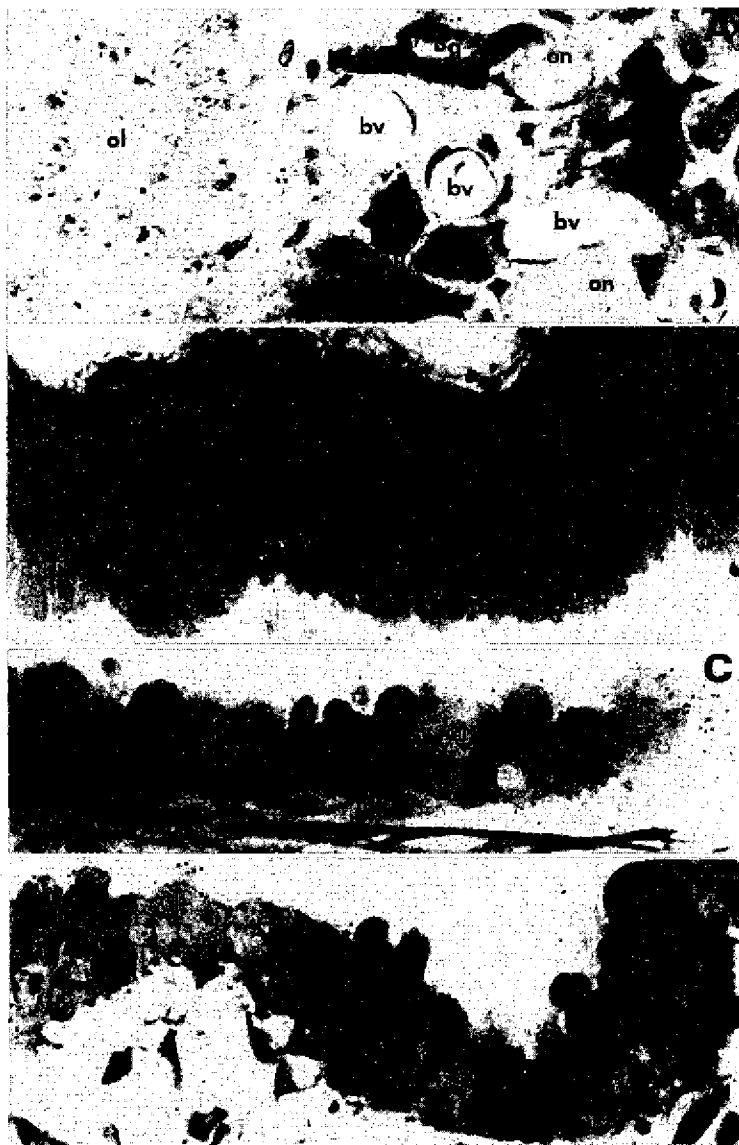
In the oesophagus, the squamous epithelium of the middle and superficial cell layers was strongly labelled, whereas the deepest (basal) layer showed fewer silver grains (Fig. 1B). Within the cells, the distribution of grains appeared to be random. In the tracheal epithelium, the mucous cells contained more bound radioactivity than the ciliated and basal cells (Fig. 1C). Both the nuclei and the cytoplasm of the cells were labelled. In the lung, bound radioactivity was present in the respiratory epithelium of the bronchi and bronchioles, whereas the pulmonary parenchyma was virtually unlabelled. The Clara cells of the bronchi and bronchioles were the major sites of binding, while the ciliated cells were more weakly labelled (Fig. 1D). Silver grains were present both over the nuclei and the cytoplasm of the cells.

#### Relation between carcinogenicity and bioactivation of NNN in target cells

There is evidence that the reactive electrophilic metabolites that induce *N*-nitrosamine-derived tumours are formed locally in the sensitive tissues (Preussmann & Stewart, 1984). One can further assume that the alkylating species are bound to cellular constituents close to the place of their formation; the intensity of the autoradiographic labelling of various cells therefore probably reflects their capacity to activate the *N*-nitrosamines. The mechanism underlying the neoplastic transformation of certain cell types is not yet known in detail, but high metabolic capacity may be one factor of great importance.

Metabolism of NNN has been demonstrated in the nasal mucosa (Brittebo & Tjälve, 1981; Brittebo *et al.*, 1983). Activation of *N*-nitrosamines is likely to involve cytochrome P450-dependent metabolism (Appel *et al.*, 1979b; Lai *et al.*, 1979). The presence of cytochrome P450 has been shown in the nasal mucosa of rats (Hadley & Dahl, 1982; Tjälve & Löfberg, 1983), and localization of cytochrome P450 and NADPH-cytochrome P450 reductase in Bowman's glands has been shown immunohistochemically (Voigt *et al.*, 1985). A strong binding of metabolites to Bowman's glands in rats has been shown for *N*-nitrosamines such as 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone (Tjälve *et al.*, 1985) and *N*-nitrosomorpholine (Löfberg & Tjälve, 1985), which are also nasal carcinogens. The malignant tumours induced by NNN in the olfactory nasal mucosa of Fischer 344 rats have been classified as carcinomas (Hoffmann *et al.*, 1975) or olfactory neuroblastomas (Hecht *et al.*, 1980a). It should be noted, however, that the histological complexity of the nasal cavity sometimes causes difficulties in discerning with certainty the

**Fig. 1.** Microautoradiographs of tissue from the olfactory region of the nose (A), the oesophagus (B), the trachea (C) and the lung and a bronchiole (D) of a Fischer 344 rat killed 4 h after a subcutaneous injection of  $^3\text{H}$ -NNN (7.4 mg/kg body weight)



In (A), there is strong labelling of Bowman's glands (Bg) in the olfactory lamina propria mucosae, whereas other structures, such as the olfactory epithelium (ol), fasciculi of olfactory nerves (on) and blood vessels (bv), are virtually unlabelled.  $\times 375$ . In (B), the middle and superficial cell layers of the oesophageal squamous epithelium is strongly labelled, while the deepest (basal) layer shows fewer silver grains.  $\times 400$ . In (C), most silver grains are localized in the mucous cells (m) of the tracheal epithelium; ci, ciliated cells.  $\times 400$ . In (D), there is greater labelling over the nonciliated Clara cells (Cl) in the bronchiole than over the ciliated cells (ci).  $\times 400$ .

cell type(s) from which the neoplasms originate. It is possible that Bowman's cells, which appear to be the cells with the highest capacity to metabolize various *N*-nitrosamines, including NNN, are commonly involved in the tumorigenesis.

A high level of bound NNN metabolites was present in the oesophageal epithelium. The oesophagus has a marked capacity to metabolize NNN (Hecht *et al.*, 1982b). Other *N*-nitrosamines, such as *N*-nitrosomethylbenzylamine (Labuc & Archer, 1982) and *N*-nitrosomorpholine (Löfberg & Tjälve, 1985), which are oesophageal carcinogens, are also metabolized in the oesophagus of rats. Cytochrome P450 has been demonstrated in rat oesophageal mucosa (Labuc & Archer, 1982). Thus, the carcinogenicity of NNN and some other *N*-nitrosamines in the oesophagus can be correlated with a local bioactivation in the sensitive cells.

The localization of bound NNN metabolites in the epithelium of the trachea and of bronchi and bronchioles of the rats contrasts with absence of tumours at these sites. The reason is not known. Conceivably, an effective repair of promutagenic lesions takes place in these tissues. A long period might also be required between DNA injuries and the occurrence of cancers at these sites, and potential neoplastic transformations would then be concealed by earlier tumours in the nasal cavity and the oesophagus. Alternatively, NNN activation in the trachea and the lung may lead to metabolites with low miscoding frequency. Activation of NNN is likely to involve an initial  $\alpha$ -hydroxylation, occurring either at the 2'- or 5'-carbon position (Hecht *et al.*, 1984). It has been shown that in target tissues such as the oesophagus and the nasal mucosa of rats there is a preference for 2'-carbon hydroxylation, which is not observed in nontarget tissues such as hamster oesophagus or rat liver (Hecht *et al.*, 1982b; Brittebo *et al.*, 1983). It is possible that this differential tissue metabolism influences the organ-specificity of the carcinogenicity.

The mucous cells of the trachea and the Clara cells of the bronchi and bronchioles, which contained the highest levels of bound NNN metabolites, have been shown to be the most strongly labelled cells in the airways of hamsters given  $^3\text{H}$ -*N*-nitrosodiethylamine (Reznik-Schüller & Hague, 1981). Cytochrome P450 has been detected in the trachea of hamsters (Löfberg & Tjälve, 1984). In the lungs, the Clara cells have been identified as the major sites of cytochrome P450 enzymes (Boyd, 1977). The lungs and the trachea are targets for the carcinogenicity by *N*-nitrosodiethylamine in hamsters (Herrold & Dunham, 1963). NNN given subcutaneously to hamsters has also been reported to induce tumours of the lung and the trachea, although at a much lower frequency than seen with 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone (Hoffmann *et al.*, 1981).

#### Acknowledgements

This study was supported by a grant from the Swedish Council for Forestry and Agricultural Research.

**CELLULAR CHANGES INDUCED BY THE  
TOBACCO-SPECIFIC CARCINOGEN  
4-(N-NITROSOMETHYLAMINO)-1-(3-PYRIDYL)-  
1-BUTANONE IN THE RESPIRATORY TRACT OF  
SYRIAN GOLDEN HAMSTERS**

M. Boutet<sup>1,3</sup>, L. Montminy<sup>1</sup> & A. Castonguay<sup>2</sup>

<sup>1</sup>*Department of Pathology and* <sup>2</sup>*School of Pharmacy, Laval University,  
Quebec, PQ G1K 7P4, Canada*

The *N*-nitrosamine 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK) is abundant in cigarette smoke and is a potent pulmonary carcinogen in Syrian golden hamsters. After intratracheal instillation of NNK to Syrian golden hamsters, we observed focal cell death in the trachea and bronchi followed by cellular regeneration. Regenerating cells were not ciliated and led to mucous-cell hyperplasia followed by squamous-cell metaplasia. Lung alterations consisted of bronchiolar hyperplasia with severe dysplastic changes in association with interstitial pneumonitis. NNK induces preneoplastic cellular changes similar to those observed in the pulmonary epithelium of smokers.

The *N*-nitrosamine NNK is abundant in cigarette smoke (0.1-0.4  $\mu\text{g}$ /cigarette; Hoffmann *et al.*, 1979). When injected subcutaneously into Syrian golden hamsters, NNK induces tracheal papillomas, bronchioalveolar carcinomas, adenocarcinomas and squamous-cell carcinomas. These changes are preceded by cellular hyperplasia and squamous-cell metaplasia (Hoffmann *et al.*, 1981). In this study, the cellular toxicity and regeneration of respiratory-tract epithelium were studied after a single intratracheal instillation of NNK. Six groups of four hamsters (weighing  $95 \pm 11$  g) were instilled with 2 mg (104  $\mu\text{mol}$ /kg) NNK dissolved in 0.2 ml 0.9% sodium chloride and were sacrificed one and three days, one, two and four weeks and three months later. Three groups of two hamsters were instilled with 2 mg/0.2 ml of NNK/0.9% sodium chloride and sacrificed 6, 12 and 18 h later. Two control groups of four hamsters were instilled with 0.2 ml 0.9% sodium chloride and sacrificed one day and four weeks later. The instillation procedure has been published (Blouin & Cormier, 1986). Tissue samples originating from the lower third of the trachea, the bronchi and the lungs were processed for light and electron microscopy.

#### **Cellular changes and regenerative processes**

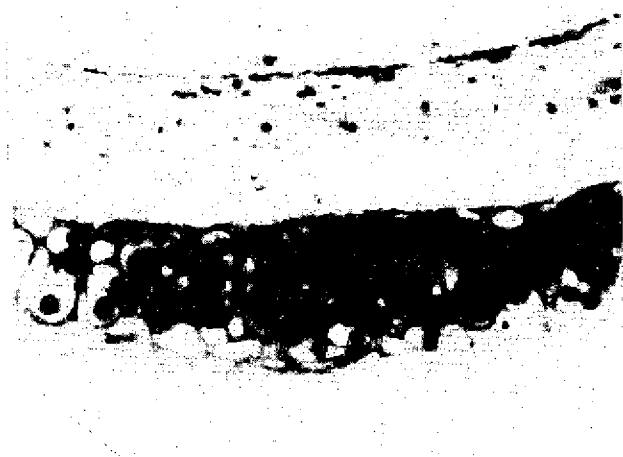
Saline-treated animals had a normal respiratory-tract epithelium, as described previously (Reznik-Schüller & Reznik, 1979). The tracheobronchial epithelium of control hamsters showed a pseudostratified cell layer with numerous columnar ciliated cells, few mucus-secreting cells and occasional basal cells. The bronchoalveolar cells contained Clara cells and pneumocytes. Shortly after NNK instillation, focal desquamation of the epithelium in the tracheobronchial tree was associated with inflammatory infiltration of

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<sup>3</sup>To whom correspondence should be addressed

polymorphonuclear cells (Fig. 1). Rapid cellular regeneration took place from the remaining basal cells, and the predominance of mucus-secreting cells (mucous-cell hyperplasia) in the regenerating area gave rise to a nonciliated epithelium (Fig. 2). Under electron microscopy, these cells showed abundant rough endoplasmic reticulum and numerous mucus granules. Their luminal surface exhibited small respiratory microvilli. Few regenerating cells were cubic, and they showed few cilia and basal corpuscles. They were joined by short desmosomes devoid of tonofilaments. After a two-week interval, regenerating cells possessed large bundles of tonofilaments and larger desmosomes. After one month, large desmosomes were more numerous, and tonofilaments were abundant. These changes corresponded to focal areas of squamous-cell metaplasia (Fig. 3). The metaplastic changes were associated with numerous areas of mucous-cell hyperplasia, which itself was associated with chronic bronchitis. Three months after NNK instillation, few foci of bronchial metaplastic changes showed severe dysplasia, and cellular proliferation had obliterated the bronchial lumina of the small bronchi (Fig. 4). The bronchiolar epithelium showed severe hyperplastic changes, thickening and severe dysplasia.

**Fig. 1. Light micrograph 12 h after instillation of NNK**



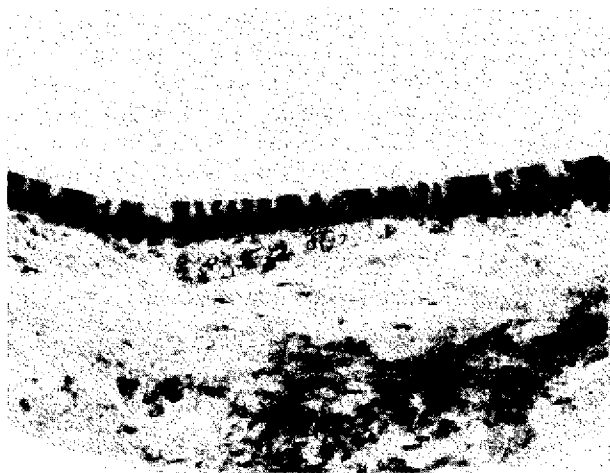
Lower third of the tracheal epithelium showing focal cellular necrosis with denuded basement membrane; note the numerous inflammatory cells.

Two weeks after NNK instillation, the alveolar epithelium and septal connective tissue showed diffuse interstitial pneumonitis (Fig. 5). The interstitial inflammatory cells were predominantly lymphocytes. Between two weeks and three months, these changes had progressed to nodular foci of cellular infiltration associated with atypical cellular changes and focal alveolar desquamation. These changes became severe at three months (Fig. 6). Pneumocytes had irregularly shaped nuclei with a prominent nucleolus; many nuclei exhibited several nucleoli. The chromatin was granulated, dense and located mainly along the nuclear membrane.

This study documents the early cellular changes that might precede the development of NNK-induced tracheobronchial and lung tumours, and might provide a better understanding of their histogenesis. Intratracheal instillation of NNK induces focal-cell death in trachea and bronchi. These early changes are followed by cellular regeneration from remaining undifferentiated basal cells. Most regenerating cells secreted mucus and were therefore not ciliated; only a few cells had a small number of cilia. These observations suggest that mucous-cell hyperplasia is an early event leading to pulmonary adenocarcinoma (Hoffmann *et al.*, 1981).

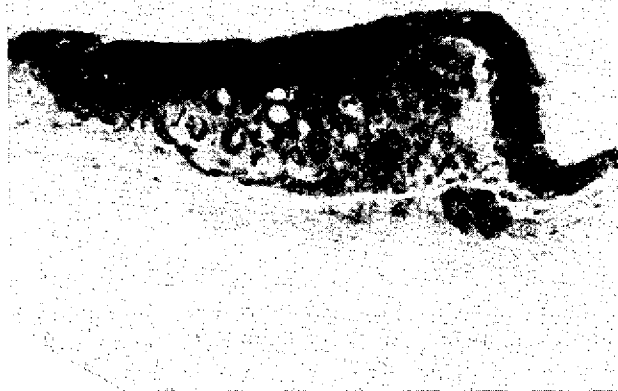


**Fig. 2. Light micrograph after 15 days**



Cellular necrosis is replaced by proliferation of cells regenerating from remaining basal cells; most of the cells are of the mucus-secreting type, devoid of cilia (deciliation)

**Fig. 3. An area of squamous-cell metaplasia can be seen in the lower third of the tracheal epithelium four weeks after instillation of NNK.**



The focal squamous-cell metaplasia observed with NNK is comparable to those observed with benzo[*a*]pyrene (Saffioti *et al.*, 1968), polonium-210 (Little & Kennedy, 1979) and cigarette smoke (Bernfeld *et al.*, 1979). As described by Hoffmann *et al.* (1981), these changes may precede squamous-cell (epidermoid) carcinoma. Peripheral lung alterations consisted of bronchiolar hyperplasia with severe dysplastic changes. Alveolar cells also showed atypical changes considered to be preneoplastic. These alterations might give rise to adenomatoid tumours, as described by Hoffmann *et al.* (1981).

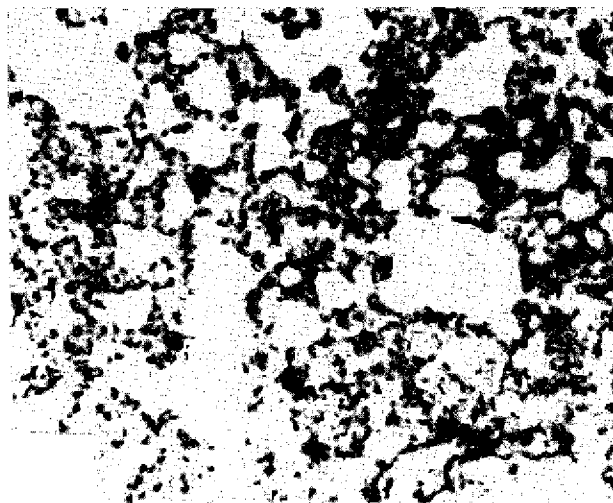
In smokers, hyperplasia of mucus-secreting cells and squamous metaplasia often precede dysplasia and carcinoma *in situ* (Spencer, 1985). Our observations with NNK-treated Syrian golden hamsters parallel this sequence of cellular change.

In conclusion, our study suggests that NNK, a potent tobacco-smoke carcinogen, induces preneoplastic cellular changes similar to those observed in the tissue of smokers. NNK may be one of the tobacco-smoke substances involved in preneoplastic changes leading to lung tumours. Furthermore, NNK may be associated with the genesis of chronic bronchitis and interstitial pneumonitis — phenomena often observed in human smokers.

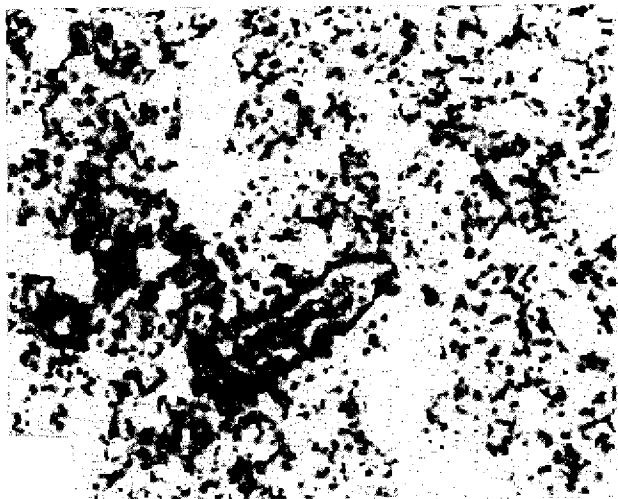
**Fig. 4.** After a three-month interval, small bronchi are obstructed by cellular proliferation of epithelial cells, often dysplastic, accompanied by a chronic inflammatory infiltration.



**Fig. 5.** One month after instillation of NNK, a mild degree of interstitial pneumonitis is associated with slightly enlarged alveolar septae showing chronic inflammatory cells.



**Fig. 6.** Three months after instillation of NNK, exaggeration of interstitial pneumonitis is observed, with slight atypical changes of the alveolar pneumocytes; the alveolar septae are enlarged, with numerous chronic inflammatory cells.



#### **Acknowledgements**

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## PATHOBIOLOGICAL EFFECTS OF ALDEHYDES IN CULTURED HUMAN BRONCHIAL CELLS

R.C. Grafström<sup>1</sup>, K. Sundqvist<sup>1</sup>, J.M. Dypbukt<sup>1</sup> & C.C. Harris<sup>2</sup>

<sup>1</sup>Department of Toxicology, Karolinska Institute, S-104 01 Stockholm, Sweden;  
and <sup>2</sup>Laboratory of Human Carcinogenesis, National Cancer Institute,  
Bethesda, MD 20892, USA

Effects of the tobacco smoke-related aldehydes, i.e., acetaldehyde, formaldehyde and acrolein, have been investigated in cultured human bronchial epithelial cells and fibroblasts. As determined from loss of colony-forming efficiency of epithelial cells, acrolein is 200- and 5000-fold more toxic than formaldehyde and acetaldehyde, respectively. The aldehydes differ markedly in their potencies to induce terminal differentiation, as indicated by cessation of growth and enhanced formation of cross-linked envelopes. The cellular content of glutathione is markedly decreased by acrolein, whereas formaldehyde and acetaldehyde only slightly decrease glutathione levels. All three aldehydes produce DNA damage, as indicated by the formation of DNA single-strand breaks and DNA protein cross-links. Both formaldehyde and acrolein are weakly mutagenic in fibroblasts. In-vitro assays of *O*<sup>6</sup>-methylguanine-DNA methyltransferase (MMT) activity indicate that it is markedly inhibited by acrolein, and to a lesser extent by formaldehyde. However, formaldehyde significantly inhibits removal of *O*<sup>6</sup>-methylguanine (*O*<sup>6</sup>-meG) in *N*-methyl-*N*-nitrosourea (MNU)-exposed cells. Thus, the many biological effects induced by aldehydes include: inhibition of proliferation, enhanced cellular differentiation, thiol depletion, DNA damage, mutation and inhibition of DNA repair in human cells. Furthermore, we speculate that exogenous or metabolically generated aldehydes may increase the genotoxicity of *N*-nitroso compounds by the dual action of causing DNA damage and inhibiting the repair of promutagenic *O*<sup>6</sup>-meG DNA lesions in human cells.

Several reactive and volatile aldehydes are found in the gaseous phase of tobacco smoke and are of interest because of their potential carcinogenicity in the human respiratory tract. In particular, formaldehyde, acrolein and acetaldehyde are present in amounts ranging from  $\mu\text{g}$  up to 1 mg per cigarette (Wynder & Hoffmann, 1979). Such aldehydes are also metabolites of xenobiotics, e.g., *N*-nitrosodimethylamine, cyclophosphamide and ethanol, and are formed endogenously as products of normal intermediary metabolism. In this study, we have investigated the effects of tobacco smoke-related aldehydes on different biological parameters, including colony survival, clonal growth, cross-linked envelope formation, content of cellular glutathione, DNA damage, mutations, and effects on *O*<sup>6</sup>-meG repair in cultured human bronchial cells.

The results of these investigations are summarized in Table 1. Clearly, acrolein was substantially more cytotoxic on a molar basis than formaldehyde, which in turn was markedly more toxic than acetaldehyde. However, mass cultures of quiescent fibroblasts maintained at confluence were found to be five- to 15-fold less sensitive to aldehyde-induced cytotoxicity when these cells were subcultured and assayed 48 h after exposure to either formaldehyde (Grafström *et al.*, 1984) or acrolein (Grafström *et al.*, unpublished data).

**Table 1. Pathobiological effects of tobacco-smoke related aldehydes in cultured human bronchial cells<sup>a</sup>**

Aldehyde	CFE <sub>50</sub> <sup>b</sup> (mM)	CGR <sub>50</sub> <sup>c</sup> (mM)	CLE <sup>d</sup>	SSB <sup>e</sup> per 10 <sup>10</sup> D	DPC <sup>e</sup> per 10 <sup>10</sup> D	GSH <sub>75</sub> <sup>f</sup> (mM)	MMT <sub>75</sub> <sup>g</sup> (mM)	6-TG <sup>f</sup> muta- tions <sup>h</sup> per 10 <sup>6</sup> survivors
Formaldehyde	0.4	0.2	6	6	70	0.8	1.0	13
Acetaldehyde	10	30	3.5	-	3	10	NT <sup>i</sup>	NT
Acrolein	2.0×10 <sup>-3</sup>	10×10 <sup>-3</sup>	11.5	1	3	8×10 <sup>-3</sup>	15×10 <sup>-3</sup>	4 <sup>h</sup>

<sup>a</sup>Data are compiled from several publications, as described in the text.

<sup>b</sup>Cells were exposed to the respective aldehyde for 1 h in serum-free growth medium. Mean colony forming efficiency (CFE) was assayed as described by Lechner *et al.* (1986), expressed as percent of control and determined from colonies containing at least 16 cells after 8 days post-treatment culture of 5000 epithelial cells per dish.

<sup>c</sup>The concentration that produced a 50% reduction in clonal growth rate (CGR) of epithelial cells after 6 h exposure; assays performed as described by Lechner *et al.* (1986)

<sup>d</sup>The mean cross-linked envelope (CLE) frequency in the total epithelial cell population after exposure to the respective aldehyde for 6 h at the concentration that inhibited clonal growth to 50%. Assays were performed as described by Willey *et al.* (1984). Values express the factor of increased CLE over control (factor 1).

<sup>e</sup>Epithelial cells were exposed as described under *b* to formaldehyde or acetaldehyde at doses that inhibited CFE to 50%. DNA damage, i.e., DNA single-strand breaks (SSB) and DNA protein cross-links (DPC) were assayed by the alkaline elution technique. Assays were performed and the number of lesions calculated as described by Kohn *et al.* (1981). Acetaldehyde produced no detectable SSB but caused DNA interstrand cross-links (Dypbukt *et al.*, unpublished data). The number of DNA lesions induced by 30 μM acrolein is shown. At CFE<sub>50</sub>, i.e., 2 μM, DNA damage was nonsignificant.

<sup>f</sup>Fibroblasts were exposed as described under *b* and assayed for their content of glutathione (GSH) as described by Grafström *et al.* (1986). GSH<sub>75</sub> indicates the concentration required to decrease the cellular GSH content to 75% of control.

<sup>g</sup>Extracts were prepared subsequent to exposure of fibroblasts to the respective aldehyde as described under *b*. MMT activity was assayed *in vitro* and expressed as percent of control, as described by Krokan *et al.* (1985). MMT<sub>75</sub> indicates the concentration required to decrease the activity to 75% of control.

<sup>h</sup>Fibroblasts were exposed to formaldehyde (100 μM) or acrolein (1 μM) for 5 h and assayed for 6-thioguanine-resistant (6-TG)<sup>f</sup> mutations, as described in detail by Grafström *et al.* (1985). Mutagenesis from exposure to acrolein was not significant (Curren *et al.*, unpublished data).

<sup>i</sup>NT, not tested

Terminal squamous differentiation of cultured epithelial cells is characterized by cessation of clonal growth and formation of cross-linked envelopes (Willey *et al.*, 1984). As assayed by the clonal growth assay, the aldehydes were all growth-inhibitory in a dose-dependent fashion. At doses that decreased the clonal growth rate, all aldehydes also markedly increased the formation of cross-linked envelopes. Furthermore, exposure of cells to the various aldehydes caused different types of DNA damage. Formaldehyde or acrolein caused higher levels of DNA protein cross-links than single-strand breaks; acetaldehyde

caused both DNA-protein and interstrand cross-links (Dypbukt *et al.*, unpublished data). Exposure of cells to aldehydes also caused a depletion of the cellular glutathione content. Acrolein was by far the most potent in this respect. All three aldehydes affected survival and glutathione content to a similar extent in both epithelial cells and fibroblasts (data not shown).

The repair of  $O^6$ -meG is catalysed by MMT and involves the direct removal and transfer of the methyl group from guanine in DNA to a cysteine-residue at the MMT protein (Yarosh, 1985). Because of the high reactivity of aldehydes towards thiols, we investigated their effects on MMT. When cell extracts were isolated subsequent to the exposure of fibroblasts to either acrolein or formaldehyde, the MMT activity was markedly inhibited by acrolein and to a lesser degree by formaldehyde (Krokan *et al.*, 1985a). When bronchial cells were initially exposed to 200  $\mu$ M MNU, an agent known to cause the formation of  $O^6$ -meG in DNA, the subsequent presence of either 100 or 300  $\mu$ M formaldehyde significantly decreased the rate of removal of  $O^6$ -meG from cellular DNA (Grafström *et al.*, 1985). Furthermore, when the mutagenic effects of formaldehyde and MNU were investigated separately and in combination, the mutation frequency was significantly greater after combined exposure to MNU and formaldehyde than after exposure to either agent alone (Grafström *et al.*, 1985). Mutagenesis after exposure to acrolein was not significant in normal fibroblasts but was clearly significant in xeroderma pigmentosum fibroblasts (Curren *et al.*, unpublished data).

Human bronchial epithelial cells or fibroblasts in serial culture provide useful in-vitro model systems for studying the pathobiological effects of tobacco smoke-related aldehydes. Acrolein, acetaldehyde and formaldehyde each cause a distinct pattern of pathobiological effects in these cells. The cytotoxic potencies of the three aldehydes differ by orders of magnitude: acrolein is more cytotoxic than formaldehyde, which in turn is more cytotoxic than acetaldehyde. Aldehydes cause terminal squamous differentiation of epithelial cells, as indicated by the decreased clonal growth rate and increased formation of cross-linked envelopes. Of several genotoxic endpoints investigated, marked effects are induced in human cells by exposure to micromolar concentrations of either acrolein or formaldehyde. Induction of differentiation, DNA damage, mutation and inhibition of DNA repair in human cells by formaldehyde seem particularly relevant because these effects occur at moderately low levels of cytotoxicity. We conclude that tobacco smoke-related aldehydes directly cause effects related to both the initiation and promotion stages of the carcinogenesis process (Grafström *et al.*, 1986). Synergistic induction of mutations by combined exposure to MNU and formaldehyde in human cells emphasizes the need for further investigation of the interactive effects of the many genotoxic components of tobacco smoke. Finally, because aldehydes are also generated endogenously from metabolism of *N*-nitrosamines, more attention should be given to the potential influence of aldehydes in *N*-nitrosamine carcinogenesis.

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# EFFECT OF CIGARETTE SMOKING AND DIETARY FACTORS ON THE AMOUNTS OF *N*-NITROSO-THIAZOLIDINE 4-CARBOXYLIC ACID AND *N*-NITROSO-2-METHYL- THIAZOLIDINE 4-CARBOXYLIC ACID IN HUMAN URINE

M. Tsuda<sup>1,5</sup>, A. Nagai<sup>1</sup>, H. Suzuki<sup>2</sup>, T. Hayashi<sup>2</sup>,  
M. Ikeda<sup>3</sup>, M. Kuratsune<sup>4</sup>, S. Sato<sup>1</sup> & T. Sugimura<sup>1</sup>

<sup>1</sup>Biochemistry Division, National Cancer Center Research Institute,  
1-1, Tsukiji 5-chome, Chuo-ku, Tokyo 104; <sup>2</sup>Tokyo Sanitarium Hospital,  
17-3 Amanuma 3-chome, Suginami-ku, Tokyo 167;

<sup>3</sup>Department of Clinical Epidemiology, University of Occupational and  
Environmental Health, 1-1, Isei-ga-oka, Yahata-Nishi-ku, Kita-Kyushu 807;  
and <sup>4</sup>Nakamura Gakuen College, 5-7-1, Befu, Johnan-ku, Fukuoka 814, Japan

The effects of cigarette smoking and dietary factors on urinary excretion of *N*-nitrosothiazolidine 4-carboxylic acid (NTCA; *N*-nitrosothioprolin) and *N*-nitroso-2-methylthiazolidine 4-carboxylic acid (NMTCA; *N*-nitroso-2-methylthioprolin) were studied in a male volunteer and in healthy Japanese subjects from the general population and Seventh-Day Adventists (SDA). Twenty-four-hour urine samples from the male volunteer were collected on 20 smoking days and 20 nonsmoking days during ingestion of a fixed diet, and the amounts of urinary *N*-nitrosamino acids were analysed by gas chromatography-thermal energy analysis. Cigarette smoking caused about two-fold (significant) increases in the amounts of NTCA and NMTCA in the volunteer. In the male subjects from the general population, not controlled for diet, the amounts of NTCA and NMTCA in 24-h urines of smokers were also significantly higher than those of the nonsmokers. The urinary excretions of NTCA and NMTCA in SDA were lower than those of nonsmokers in the general population. It was concluded that cigarette smoking is one of the important factors in determining the amounts of NTCA and NMTCA in human urine. Dietary factors also apparently influence the urinary levels of these *N*-nitrosamino acids. In addition, an apparent sex difference in the urinary excretion of NTCA and NMTCA (about two-fold higher in females) was observed in the general population but not in SDA. The *N*-nitrosoproline (NPRO) level was significantly higher in SDA than in nonsmokers in the general population.

Since Ohshima and Bartsch (1981) reported endogenous formation of NPRO in the human body and its urinary excretion, urinary levels of NPRO have been considered to be a good index of endogenous nitrosation in the human body. In addition, Ohshima *et al.* (1983) and Tsuda *et al.* (1983) found NTCA and *trans*- and *cis*-NMTCA in human urine. These compounds as well as NPRO, were shown to be major and commonly occurring *N*-nitroso compounds in human urine (Ohshima *et al.*, 1984a; Wagner *et al.*, 1985; Lu *et al.*, 1986; Tsuda *et al.*, 1986). NTCA is not mutagenic to *Salmonella typhimurium* TA98, TA100 or

<sup>5</sup>To whom correspondence should be sent

TA104 with or without metabolic activation (Tahira *et al.*, 1984; Umamo *et al.*, 1984) and is expected to be noncarcinogenic like NPRO (Mirvish *et al.*, 1980a). The rate of in-vitro nitrosation of thioproline at pH 2 is about 1000 times faster than that of proline (Tahira *et al.*, 1984), and thus thioproline is considered to be an effective nitrite trapping agent.

In this study, to determine the levels of urinary NPRO, NTCA and NMTCA in the general population in Japan ingesting an ordinary diet, and the effects of cigarette smoking and dietary factors on the levels of these *N*-nitrosamino acids, we analysed 146 samples of 24-h urine collected from a male volunteer and from 26 other subjects from the healthy general population and from 36 healthy SDA.

### Volunteer

The volunteer (male, 45 years) smoked 15-20 filtered cigarettes per day for 20 nonconsecutive days and did not smoke for another 20 nonconsecutive days on a fixed diet (Tsuda *et al.*, 1986), and his 24-h urine samples were collected. Other 24-h urine samples were collected during 20 nonsmoking days on an unrestricted diet.

### Subjects in the general population

The male subjects consisted of eight smokers (average, 20 cigarettes/day; mean and range of age, 35, 22-45 years) and ten nonsmokers (age, 33, 12-45). Most of the eight female subjects were housewives (age, 30, 10-37). All subjects ingested their usual diets and had their usual amounts of alcohol and/or cigarettes, and did not take ascorbic acid, except in their diet, on the days of 24-h urine collection.

### Japanese SDA

Japanese SDA examined were workers at the Tokyo Sanitarium Hospital (Suginami, Tokyo) and members of their families, and were all strict lacto-ovo vegetarians. They consisted of 18 men (age, 39, 13-54) and 18 women (age, 42, 23-76), and were all nonsmokers. On the days of 24-h urine collections they did not take any alcohol, coffee or black tea. They took rice, eggs, milk and milk products, and more vegetables, cereals, soya-bean products, gluten and green tea than the general population. Their diets were not restricted on the days of urine collection.

### Amounts of urinary NTCA and NMTCA

*Effect of cigarette smoking:* Cigarette smoking caused significant increases (about two fold) in the amounts of NTCA and NMTCA in 24-h urine samples of the male volunteer (Table 1 and Fig. 1; Tsuda *et al.*, 1986). The quantities of NTCA and NMTCA in cigarette smoke were not more than 5 ng per cigarette for each compound. Thus, the enhancement by smoking was probably due to in-vivo formation of NTCA and NMTCA.

The amounts of urinary NTCA and NMTCA were also significantly higher in smokers than in nonsmokers in the general male population ( $p < 0.05$  and  $p < 0.02$ , respectively, Table 1). Higher levels of urinary NTCA and NMTCA in smokers than in nonsmokers were reported earlier by Ohshima *et al.* (1984a), by Lu *et al.* (1986) and by our own group (Tsuda *et al.*, 1986). Since placing smokers and nonsmokers on the same diet results in significantly higher urinary excretion of NPRO (Hoffmann & Brunnemann, 1983), it is likely that the differences in the urinary excretion of the three *N*-nitrosamino acids observed in the two groups would have been even higher if both groups had had the same diet. It is known that cigarette smokers consume more alcohol than nonsmokers and that there are also



**Table 1. *N*-Nitrosamino acids in 24-h urine samples from a male volunteer, from subjects from the general population and from Seventh-Day Adventists in Japan**

Population <sup>a</sup>	No. urine samples/ no. urine donors	$\mu\text{g}/24\text{-h urine, mean} \pm \text{SD}^b$ (range)			
		NPRO	NTCA	NMTCA <sup>c</sup>	Total NAA <sup>d</sup>
<b>Volunteer</b>					
FIX-S	20/1	1.8 $\pm$ 0.9 (0.4 - 4.1)	8.7 $\pm$ 4.6 (2.7 - 21.3)	8.5 $\pm$ 4.1 (2.9 - 19.0)	19.0 $\pm$ 6.5 (9.1 - 28.5)
FIX-NS	20/1	1.1 $\pm$ 0.5 (0.3 - 2.6)	3.9 $\pm$ 1.1 (1.8 - 6.0)	5.6 $\pm$ 1.9 (2.4 - 9.2)	10.6 $\pm$ 2.8 (5.9 - 14.7)
URD-NS	20/1	1.4 $\pm$ 0.8 (0.7 - 3.6)	7.9 $\pm$ 6.4 (2.2 - 24.9)	8.7 $\pm$ 6.4 (1.1 - 27.6)	18.0 $\pm$ 12.4 (4.6 - 48.9)
<b>General population</b>					
Male NS	19/10	1.6 $\pm$ 1.1 (0.6 - 4.5)	6.4 $\pm$ 5.5 (0.7 - 22.9)	5.4 $\pm$ 3.6 (0.4 - 14.5)	13.4 $\pm$ 7.2 (2.0 - 30.9)
Male S	17/8	2.2 $\pm$ 1.2 (0.8 - 3.9)	10.5 $\pm$ 6.5 (3.6 - 23.1)	8.8 $\pm$ 4.1 (1.4 - 14.9)	21.5 $\pm$ 9.9 (7.6 - 39.7)
Female NS	14/8	1.9 $\pm$ 0.9 (0.8 - 3.9)	12.1 $\pm$ 5.9 (3.0 - 20.7)	10.2 $\pm$ 7.9 (1.5 - 24.7)	24.1 $\pm$ 11.4 (5.8 - 40.6)
<b>SDA</b>					
Male NS	18/18	4.3 $\pm$ 6.0 (0.3 - 24.4)	5.1 $\pm$ 3.4 (1.2 - 11.7)	3.6 $\pm$ 2.9 (0.4 - 11.7)	13.1 $\pm$ 10.8 (2.3 - 47.2)
Female NS	18/18	3.0 $\pm$ 3.2 (0.2 - 12.6)	5.2 $\pm$ 4.8 (0.6 - 19.4)	3.4 $\pm$ 3.7 (0.4 - 13.8)	11.6 $\pm$ 10.0 (1.4 - 34.2)

<sup>a</sup>FIX-S, fixed diet with smoking; FIX-NS, fixed diet without smoking; URD-NS, unrestricted diet without smoking; NS, nonsmokers; S, smokers

<sup>b</sup>Collection of urine and analysis of *N*-nitrosamino acids by gas chromatography-thermal energy analysis were performed as described previously (Tsuda *et al.*, 1986)

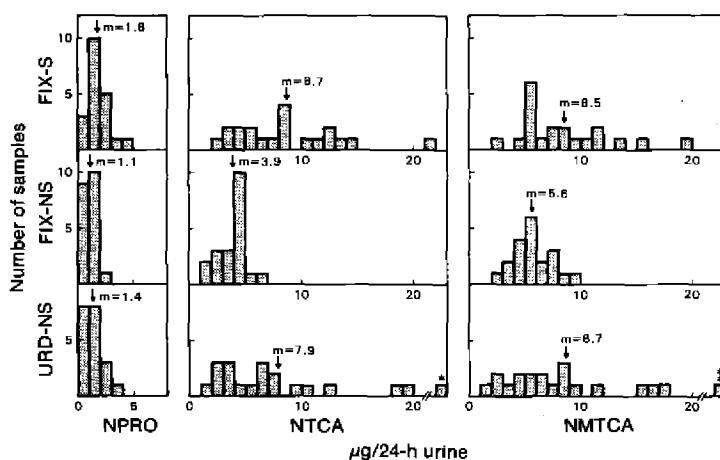
<sup>c</sup>Sum of *trans*- and *cis* isomers of NMTCA

<sup>d</sup>Sum of three *N*-nitrosamino acids

differences in food intake (Matsuya, 1982; Schottenfeld & Fraumeni, 1982; Fisher & Gordon, 1985).

Cigarette smoke contains large amounts of aldehydes, nitrogen oxides and hydrogen cyanide (Wynder & Hoffmann, 1979). Formaldehyde and acetaldehyde react readily with cysteine to form thioproline and methylthioproline, respectively; therefore, the presence of these aldehydes in cigarette smoke may participate in in-vivo formation of these sulfur-containing amino acids (Braven *et al.*, 1967). In-vivo formation of *N*-nitrosomorpholine in rodents exposed by inhalation to nitrogen dioxide and gavaged with morpholine was also reported (Mirvish *et al.*, 1981; Van Stee *et al.*, 1983). Hydrogen cyanide is a precursor of

Fig. 1. Effects of cigarette smoking and dietary factors on urinary excretion of N-nitrosamino acids in a male volunteer



Abbreviations: see footnotes to Table I; m, mean value (see Table I); \*, 24.9 µg/24-h urine; \*\*, 27.6 µg/24-h urine. The significance of differences between values for groups was examined by Student's *t*-test (one-tailed): FIX-S vs FIX-NS: NPRO,  $p < 0.005$ ; NTCA,  $p < 0.0005$ ; NMTCA,  $p < 0.005$ . FIX-NS vs URD-NS: NTCA,  $p < 0.005$ ; NMTCA,  $p < 0.025$

thiocyanate, which is an effective catalyst of nitrosation (Mirvish, 1975). Thus, cigarette smoking accelerates the nitrosation of thioproline and methylthioproline in the stomach by increasing the salivary level of thiocyanate (Tannenbaum *et al.*, 1981; Ladd *et al.*, 1984b).

*Effects of dietary factors:* The amounts of urinary NTCA and NMTCA in SDA were lower than those in male and female nonsmokers in the general population. Alcohol drinking increases the serum level of acetaldehyde (Korsten *et al.*, 1975), and this may increase the urinary level of NMTCA *via* formation of methylthioproline. SDA take no alcohol, and this may partially explain their lower excretion of NMTCA.

Furthermore, the SDA who collaborated in this study did not take any fish or meat. Fish is an important protein source for the general population of Japan. Fish of the cod family, which is commonly eaten in Japan, are known to produce high amounts of formaldehyde and dimethylamine during storage due to oxidative degradation of trimethylamine oxide, an abundant component of their tissues (Amano & Yamada, 1964). Therefore, ingestion of cod and their products contributes to the high level of urinary NTCA in the general population, because formaldehyde is a precursor of thioproline. In addition, preformed NTCA has also been detected in broiled meat (mutton) and bacon (Helgason *et al.*, 1984; Sen *et al.*, 1985).

SDA probably ingest fewer aldehydes, thioproline and methylthioproline and preformed NTCA than Japanese in the general population. In addition, SDA were apparently less exposed to gaseous pollutants such as aldehydes, nitrogen oxides and hydrogen cyanide present in cigarette smoke, even through passive smoking, because they usually live in areas where people do not smoke. These factors may explain the lower levels of NTCA and NMTCA in SDA, although their nitrate intake through vegetables is expected to be high.

It is noteworthy that Lu *et al.* (1986) recently reported that Chinese populations have very low levels of urinary NMTCA (less than 1  $\mu\text{g}/\text{day}$ ) — much lower than that found in the present study. The explanation for this difference should be explored in future studies.

*Sex difference:* An interesting finding in this study was that the urinary amounts of NTCA and NMTCA in female subjects are about two-fold higher than those in male subjects among nonsmokers in the general population ( $p < 0.005$  and  $p < 0.025$ , respectively), but not in SDA. One possibility is that women might ingest more vegetables than men and so their nitrate intake is higher. However, this factor alone does not seem to explain the sex difference, because none was observed in the level of NPRO. Other physiological factors, including metabolic differences (such as cellular thiol level; Igarashi *et al.*, 1983), may also contribute to this sex difference.

*Urinary level of NPRO:* The amounts of urinary NPRO in SDA ( $3.4 \pm 4.7 \mu\text{g}/\text{day}$ , male plus female) was significantly higher than that of nonsmokers in the general population ( $1.7 \pm 1.0$ , male plus female,  $p < 0.025$ ). Six out of 36 SDA subjects excreted more than 5  $\mu\text{g}/\text{day}$  NPRO (5.9, 9.2, 10.5, 12.4, 12.6 and 24.4  $\mu\text{g}/\text{day}$ ). In contrast, none of the 26 subjects in the general population, even the smokers, were found to excrete more than 5  $\mu\text{g}/\text{day}$  NPRO. It is noteworthy that Stich *et al.* (1984a) found that the average urinary NPRO levels of 22 vegetarians and 14 lacto-vegetarians were 0.8 and 1.4  $\mu\text{g}/24 \text{ h}$ , respectively, and that these values are noticeably lower than the levels of Japanese SDA in our study.

### Conclusion and future aspects

In this study, we found that the levels of urinary NPRO, NTCA and NMTCA in the nonsmoking general population in Japan were  $1.6 \pm 1.1$ ,  $6.4 \pm 5.5$  and  $5.4 \pm 3.6 \mu\text{g}/\text{day}$  for men and  $1.9 \pm 0.9$ ,  $12.1 \pm 5.9$  and  $10.2 \pm 7.9 \mu\text{g}/\text{day}$  for women, respectively. We also showed that cigarette smoking and dietary factors significantly influence the urinary amounts of these *N*-nitrosamino acids. However, in an experiment on a volunteer (Fig. 1), we found that the urinary amounts of these *N*-nitrosamino acids were relatively constant when the diet was fixed and the subject did not smoke compared with those on an unrestricted diet; we could therefore show clearly that cigarette smoking increased the amounts (Tsuda *et al.*, 1986). Thus, measurements of urinary NTCA and NMTCA should provide sensitive parameters of endogenous formation of *N*-nitroso compounds, not only in normal and achlorhydric stomachs but also at other sites such as the lung. Their measurements might be useful in evaluating the effects of exposure to nitrogen oxides through air pollution and passive smoking, and also the effects of nitrate generated endogenously from ammonia by the oxidative action of macrophages (Stuehr & Marletta, 1985).

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## EFFECTS OF AIR-CURING ENVIRONMENT ON ALKALOID-DERIVED NITROSAMINES IN BURLEY TOBACCO

R.A. Andersen<sup>1</sup>, H.R. Burton<sup>2</sup>, P.D. Fleming<sup>1</sup>, T.R. Hamilton-Kemp<sup>2</sup> & S.L. Gay<sup>2</sup>

<sup>1</sup>United States Department of Agriculture and <sup>2</sup>College of Agriculture,  
University of Kentucky, Lexington, KY 40546, USA

Levels of nitrite and pyridine alkaloid-derived total tobacco-specific nitrosamines (TSNA) were significantly higher in tobacco leaf (normal or late harvest) air-cured at 32°C/83% relative humidity (RH) than in more moderate environments, i.e., 15°C/50% RH and 24°C/70% RH. These constituents increased appreciably from day 10 to day 21 of the cure. The near-concurrent appearances of maximal total contents of TSNA [sum of *N*'-nitrosonornicotine (NNN), *N*'-nitrosoanatabine (NAT), *N*'-nitrosoanabasine (NAB) and 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK)] and nitrite supports the concept that nitrite is a limiting and proximal precursor of total TSNA during the curing of tobacco. During a long curing period (50 days) at 32°C/83% RH, nicotine and anatabine contents decreased, but TSNA contents increased in leaves of all harvest dates and stalk positions. These results support the view that nicotine and anatabine are precursors of TSNA. Measurement of nitrite and individual alkaloids during post-harvest processing of tobacco leaf may provide an index of the potential accumulation of alkaloid-derived nitrosamines.

Burley and other air-cured tobaccos are widely used as major components in cigarette blends, in many types of smokeless tobacco products, in pipe and cigar tobaccos, and in black cigarettes. Burley tobacco leaves have characteristically high concentrations of the TSNA precursors, nitrate and nicotine, which vary with stalk position. NNN and NNK are present at low concentrations (< 3 µg/g) in burley tobacco after conventional air curing and at increasing levels (sometimes > 50 µg/g) in the same tobacco cultivars during successive stages of homogenized leaf curing (HLC; Andersen *et al.*, 1982; Andersen & Kemp, 1985). The purpose of this investigation was to determine the effects of temperature- and humidity-controlled air-curing environments on the concentrations of alkaloid-derived nitrosamines and their precursors in a burley tobacco cultivar sampled at frequent intervals during curing in a range of environments representative of conventional air curing.

### Growth, curing and sampling of tobacco

*Nicotiana tabacum* L. cv. Ky 14 was grown in 1984 and 1985 by standard agronomic practices. Plants grown in 1984 were harvested four weeks (H-1-84) and seven weeks (H-2-84) after topping, and the sticks of tobacco were placed in controlled environment chambers. Ten H-1-84 plants, each with three replicates, were maintained in a separate chamber for 21 days at one of three sets of constant temperature/RH conditions, i.e., low, 15°C/50% RH; medium, 24°C/70% RH; high, 32°C/83% RH, which caused identical moisture losses for tobacco lamina. After chamber curing, H-1-84 plants were stored in a

curing barn for ten weeks at ambient conditions. Average high, low and mean temperatures during this period were 15.6°C, 6.1°C and 11.1°C, respectively, and average high and low RHs were 96% and 61%. Ten H-2-84 plants, each with three replicates, were maintained for 50 days in chambers at one of the three sets of conditions used for H-1-84 plants. After chamber curing, plants were transferred to a barn for four weeks at average high, low and mean temperatures of 8.5°C, -1.7°C and 3.3°C, respectively, and average high and low RHs of 92% and 54%. After barn storage, tobacco leaves undergoing the various treatments were removed and separated into equivalent numbers of leaves from upper, middle and lower stalk positions. Lamina were separated from mid-veins. Samples were ground and then stored at -20°C until analysed.

Plants grown in 1985 were harvested one, four and seven weeks after topping for H-1-85, H-2-85 and H-3-85, respectively. Three replicates of harvested tobaccos were then chamber-cured, under the same conditions used for 1984 tobacco at high-temperature/RH conditions. After curing for 21 days, the leaves were prepared as described above. Additionally, three replicates were chamber-cured at 32°C/83% RH for 50 days and then prepared in the same way.

### Chemical analyses

Nitrite and calcium were determined as described previously (Andersen *et al.*, 1982). Nitrite was also determined by a previously described modification (Andersen & Kemp, 1985) of the method of Sen and Donaldson (1978). Individual alkaloids (nicotine, normicotine, anatabine and anabasine) were determined by the procedure of Severson *et al.* (1981).

NNN, NNK, NAT and NAB were determined by the capillary gas chromatographic-nitrogen-phosphorus detector (GC-NPD) procedure previously described for NNN and NNK (Andersen & Kemp, 1985), modified to include NAT and NAB. NAT and NAB were synthesized by nitrosation of anatabine and anabasine, respectively, in a manner described for the nitrosation of morpholine (Lijinsky & Taylor, 1975b); anatabine was synthesized by the method of Quan *et al.* (1965). GC-mass spectrometric analyses were carried out to determine peak identities in samples with a Finnegan 705 Ion Trap Detector, using previously described GC conditions (Andersen & Kemp, 1985).

### GC-NPD methods for NNN and NNK extended to NAT and NAB

NNN, NAT, NAB and NNK were well separated from each other and from the azobenzene internal standard. Relative retentions were determined. 2,3'-Dipyridyl, cotinine, *N'*-formylnornicotine and other pyridine acyl alkaloids were also present in sample extracts. Quantification of each nitrosamine was based on the use of a recovery-response factor determined for each authentic nitrosamine carried through the analytical procedure (Andersen & Kemp, 1985).

### Effects of curing on composition of 1984 tobacco

Nitrite contents were significantly higher in cured 1984 leaf lamina (normal or late harvest) from the 32°C/83% RH environment than from the more moderate environments (Table 1). A maximal increase of 1460 µg/g (> 3000 fold) was observed in late-harvested middle-leaf lamina. Similar increases of nitrosamine levels occurred: the following multiples of increase were seen from levels in the milder environments: NNN, × 116; NAT, × 104 and NNK, × 248. The largest increases in nitrite and nitrosamines were in leaves from top and middle stalk positions. Correlations ( $r$ ;  $p < 0.1$ ) of nitrite contents in lamina harvested at the normal time with levels of NNN, NAT and NNK were 0.65, 0.74 and 0.64, respectively. In

late-harvested lamina, the corresponding  $r$  values ( $p < 0.01$ ) were 0.88, 0.97 and 0.94. Correlations ( $p < 0.05$ ) of NNN with NAT and NNK in the cured lamina from tobacco harvested at the normal time were 0.87 and 0.72, respectively. Corresponding  $r$  values ( $p < 0.01$ ) in late-harvested leaf were 0.99 and 0.97.

**Table 1. Effects of temperature and RH during curing on accumulation of nitrite and alkaloid-derived nitrosamines in 1984 cured leaf lamina**

Leaf stalk position	°C/% RH	Nitrite ( $\mu\text{g/g}$ )	Nitrosamine ( $\mu\text{g/g}$ )		
			NNN	NAT	NNK
Harvested 4 weeks after topping and chamber-cured 20 days					
Top	15/50	<i>b</i>	4.4	20.7	0.1
	24/70	-	2.5	8.2	-
	32/83	0.9	7.3	34.1	0.8
Middle	15/50	2.0	5.0	20.1	1.2
	24/70	-	1.6	17.8	-
	32/83	15	13.5	42.2	1.5
Bottom	15/50	0.2	2.3	17.8	0.1
	24/70	-	1.8	7.4	0.1
	32/83	28	7.8	39.3	1.0
Harvested 7 weeks after topping and chamber-cured 50 days					
Top	15/50	0.7	5.5	23.0	0.3
	24/70	0.3	3.0	8.9	0.3
	32/83	1392	481	1697	64.1
Middle	15/50	0.8	5.3	23.0	0.5
	24/70	-	3.0	8.9	0.3
	32/83	1460	426	1200	37.4
Bottom	15/50	0.8	5.0	17.8	0.8
	24/70	0.8	2.3	11.9	0.3
	32/83	371	46.3	134	4.6
LSD:05 <sup>a</sup>		0.3	2.3	7.4	0.8

<sup>a</sup>LSD:05, least significant difference ( $p < 0.05$ )

*b* -, not detected

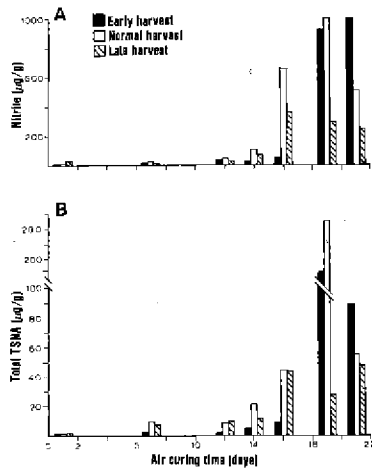
The higher levels of nitrosamines in the leaves from late-harvested plants can be explained on the basis of the longer curing period used for these plants. Our results indicate that accumulations of high TSNA concentrations can be avoided by air curing at 24°C/70% RH or less.

#### Effects of curing on the composition of 1985 tobacco

Nitrite concentrations in top-leaf lamina from each harvest increased appreciably during the last ten days of curing at 32°C/83% RH (Fig. 1A); maximal concentrations occurred at 16-21 days. Concentrations of total TSNA (sum of NNN, NAT, NAB and NNK) also increased in lamina from the top-stalk position of each harvest during the last ten days of

curing under these conditions (Fig. 1B). NAB was not detectable. Maximal TSNA contents were present at 16-21 days. The near-concurrent appearance in lamina of maximal total TSNA and maximal nitrite in the three tobaccos supports the concept that nitrite is one of the limiting precursors of TSNA. A lag was observed previously in maximal nitrosamine accumulation with respect to nitrite observed during HLC (Andersen & Kemp, 1985); this trend was not apparent in the present experiments. The correlation between nitrite and TSNA in lamina, calculated over the three harvest dates, was 0.85 ( $p < 0.001$ ).

**Fig. 1. A, Nitrite and B, total TSNA accumulating in top-leaf lamina of tobacco during air curing at 32°C/83% RH**



The effects of a long curing period (50 days) at 32°C/83% RH on nitrosamine accumulations and changes in alkaloid content in lamina are summarized in Table 2. Nicotine and anatabine levels decreased during the cure, but nornicotine and anabasine contents did not. The contents of all nitrosamines were increased significantly in lamina among all harvest dates and leaf-stalk positions. Individual alkaloids in lamina before curing occurred at high concentrations at later harvest dates. After curing, alkaloid levels were similar among samples harvested at different times. Nitrosamine contents, however, were higher at late harvest than at earlier harvests. The finding of highest concentrations of precursor alkaloids in late-harvested tobacco during initial stages of curing may account for this. The highest nitrosamine levels in lamina were usually in those from top or middle leaf positions. The correlation values of the changes in nicotine or anatabine contents with the changes in total TSNA content were  $-0.62$  ( $p < 0.5$ ) and  $-0.59$  ( $p < 0.1$ ), respectively. The results support the view that nitrite, nicotine and anatabine are major precursors of TSNA.

### Acknowledgements

We are indebted to Dr L. Bush and Dr M. Djordjevic for advice, Mr T. Becherer and Mrs S. Mojesky for technical help and Ms S. Blakemore for manuscript preparation.

**Table 2. Effect of curing for 50 days at 32°C/83% RH on contents of individual alkaloids and nitrosamines in 1985 leaf lamina**

Harvest date <sup>a</sup>	Leaf stalk position	Component: after cure (before cure)						
		Nicotine (mg/g)	Nornicotine (mg/g)	Anatabine (mg/g)	Anabasine (mg/g)	NNN (µg/g)	NAT (µg/g)	NNK (µg/g)
Early	Top	6.9 (17.8)	1.58 (0.45)	0.69 (0.79)	0.15 (0.06)	50.2 (0.2)	148.2 (0.7)	0.9 (0.1)
	Middle	4.0 (22.0)	0.97 (0.58)	0.48 (0.91)	0.08 (0.08)	46.0 (0.2)	160.1 (0.7)	1.2 (0.1)
	Bottom	4.0 (22.0)	1.32 (0.53)	0.68 (0.81)	0.11 (0.08)	20.4 (0.2)	83.7 (0.7)	1.2 (0.1)
Normal	Top	4.9 (38.0)	0.91 (0.84)	0.74 (1.90)	0.12 (0.19)	30.9 (0.2)	226.7 (1.5)	1.0 (0.1)
	Middle	3.2 (28.5)	0.61 (0.85)	0.56 (1.72)	0.16 (0.17)	38.2 (0.2)	295.7 (0.7)	1.9 (0.1)
	Bottom	1.6 (22.4)	0.73 (0.40)	0.39 (1.47)	0.12 (0.10)	22.9 (0.2)	165.2 (0.7)	1.4 (0.1)
Late	Top	5.2 (49.0)	1.55 (1.86)	0.84 (1.80)	0.27 (0.27)	96.4 (0.2)	372.0 (1.5)	2.6 (0.1)
	Middle	5.0 (38.0)	1.20 (1.52)	0.63 (2.15)	0.21 (0.21)	171.3 (0.2)	557.2 (0.7)	7.6 (0.1)
	Bottom	3.9 (39.0)	1.69 (1.54)	0.63 (2.97)	0.22 (0.26)	133.7 (0.2)	240.7 (0.7)	3.0 (0.1)
LSD:05 <sup>b</sup>		2.5	0.72	0.29	0.11	2.3	7.4	0.5

<sup>a</sup>Early, normal and late = 1, 4 and 7 weeks after topping, respectively

<sup>b</sup>LSD:05, least significant difference ( $p < 0.05$ )



## A STUDY OF SNUFF CARCINOGENESIS

K.D. Brunnemann, A. Rivenson, J.D. Adams, S.S. Hecht & D. Hoffmann

*American Health Foundation, Naylor Dana Institute for Disease Prevention,  
Dana Road, Valhalla, NY 10595, USA*

Dry snuff contains high levels of tobacco-specific *N*-nitrosamines (TSNA); their concentrations exceed by more than 100 times the quantities of nitrosamines found in any other consumer product. The concentrations of TSNA are similar in dry snuff and in the more popular moist snuff. In addition to the four TSNA identified earlier [*N*'-nitrosoornicotine (NNN), 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK), *N*'-nitrosoanatabine (NAT) and *N*'-nitrosoanabasine (NAB)], two new nitrosamines were detected in snuff, namely 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanol (NNAl; 0.07-0.15 ppm) and 4-(*N*-nitrosomethylamino)-4-(3-pyridyl)-1-butanol (iso-NNAl; 0.06-1.1 ppm). After oral swabbing with a mixture of NNN and NNK, rats developed tumours of the oral cavity and lung, showing that these TSNA are not only organ-specific carcinogens but can also induce local tumours. After swabbing an extract of snuff containing the same concentrations of NNN and NNK, significantly fewer tumours were induced in the oral cavity and lung, indicating inhibition of the tumorigenic activity of the TSNA by other snuff constituents.

Snuff dipping has increased significantly in the USA and in Scandinavian countries, especially among young people. This habit is associated causally with cancer of the oral cavity. So far, exposing animals to snuff has generally resulted in little or no tumour production (IARC, 1985; US Department of Health and Human Services, 1986). However, snuff contains at least three types of known carcinogens — polynuclear aromatic hydrocarbons, polonium-210 and *N*-nitrosamines. The TSNA occur in at least 100 times the quantities found in other consumer products (Hoffmann *et al.*, 1986a,b). Previous studies have shown that two TSNA are powerful organ-specific carcinogens (Hoffmann & Hecht, 1985). In this communication, we report on the concentration of TSNA in moist and dry snuff, on the identification of two additional nicotine-derived *N*-nitrosamines, and on the results of bioassays on the carcinogenicity of NNN plus NNK and of snuff in the oral cavity of rats.

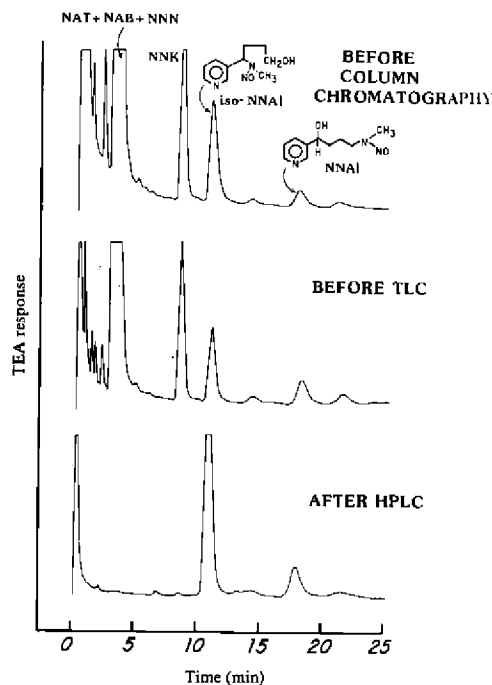
### Analysis of TSNA in snuff

For the determination of TSNA in snuff by gas chromatography-thermal energy analysis (GC-TEA), we use a chromatographic system employing a 4-m column packed with 10% UCW-982 (Adams *et al.*, 1983). More recently, we have chosen a 2-m column packed with 3% XE-60 to allow the analysis of later-eluting nitrosamines (Brunnemann *et al.*, 1986). For the large-scale isolation, we extracted 680 g of snuff tobacco with citrate-phosphate buffer containing ascorbic acid. After 2 h, the slurry was filtered over Celite 545 and the filtrate was then saturated with sodium chloride and extracted with dichloromethane. This extract was dried (sodium sulfate), concentrated to 10 ml and then chromatographed on 200 g silica gel with 500 ml dichloromethane (fraction 1, discarded). The eluting solvent was then changed to dichloromethane:methanol 10:1, and 20-ml fractions were collected. Each

fraction was analysed by GC-TEA using a 2-m × 6.4-mm (2 mm id) glass column packed with 3% XE-60 on Gas Chrom Q at an oven temperature of 200°C.

According to retention times on GC-TEA, fractions 4-6 showed the presence of NNAl and iso-NNAl (Fig. 1). For the preparation of reference standards, NNAl was synthesized according to published methods (Hecht *et al.*, 1980b) and iso-NNAl was obtained by reduction of 4-(*N*-nitrosomethylamino)-4-(3-pyridyl)butanal (NNA) with sodium borohydrate (Hecht *et al.*, 1977).

**Fig. 1. Chromatographic enrichment of snuff extract**



TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; TEA, thermal energy analyser

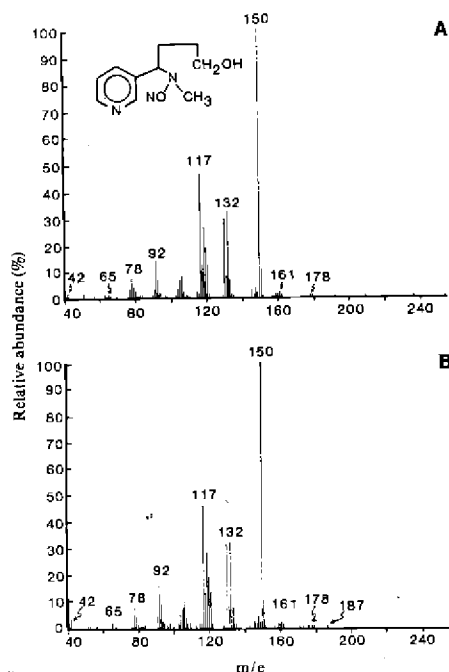
Fractions 4-6 were combined and further enriched (preparative TLC and reverse-phase HPLC, yielding a relatively clean subfraction (Fig. 1, bottom). This fraction was then analysed by GC-mass spectrometry using a 15 m × 0.32 mm id DB-5 capillary column (splitless), temperature programmed from 100-200°C. Under these conditions, iso-NNAl and NNAl had retention times of 11.5 and 13.7 min, respectively.

Figure 2 shows the identity of iso-NNAl isolated from snuff as compared to reference material. The mass spectral identification of NNAl has not been completed yet; however, applying different chromatographic conditions (packed and capillary GC, TLC and HPLC), we have supportive evidence as to its identity.

For the analysis of snuff samples (both moist and dry), the analytical procedure was slightly modified. Samples of 20-30 g of snuff were extracted as described above; the extract was chromatographed on 60 g silica gel with 200 ml dichloromethane (discarded), 100 ml dichloromethane:methanol 20:1 (discarded) and finally dichloromethane:methanol 2:1. The latter fraction was dried, concentrated to 5 ml and analysed by GC-TEA using an XE-60 column, as described previously. For better resolution of NAT and NAB, each sample was also analysed on a UCW-982 column (Adams *et al.*, 1983).

Table 1 lists the data for TSNA, including iso-NNAl, as well as the moisture and alkaloid levels; the latter were analysed using a previously published method (Hoffmann *et al.*, 1986b). All data are expressed in units per gram of wet snuff; we felt that this would permit direct comparison of dry and moist snuff products. Since mass spectral identification of NNAl is still pending, we have so far determined the NNAl concentration in only two moist snuff brands, D (0.15 ppm) and E (0.07 ppm).

**Fig. 2. Mass spectral identification of iso-NNAl; A, reference; B, isolated from snuff tobacco**



Moist snuff amounts to about 80% of the total snuff sales in the USA. We have analysed three popular brands of dry snuff because four case-control studies showing a strong association between snuff dipping and oral cancer relate to the use of this type of snuff (Winn *et al.*, 1981; IARC, 1985). As reported here, the nitrosamine levels in the three samples of dry snuff compare in order of magnitude with those obtained for five popular brands of moist snuff, as listed in Table 1 and reported elsewhere (US Department of Health and Human Services, 1986).

The newly identified NNAl is a known carcinogen (Hoffmann & Hecht, 1985) and iso-NNAl is currently being bioassayed for carcinogenicity in mice and rats.

#### Bioassays for carcinogenicity

NNN and NNK induce tumours of the nasal cavity, lung, oesophagus and liver in mice, rats and hamsters (Hoffmann & Hecht, 1985). Since snuff dipping is associated with oral cancer in humans, and since saliva extracts contain TSNA from tobacco during

**Table 1. Moisture content, alkaloids and tobacco-specific *N*-nitrosamines in US commercial brands of dry and moist snuff<sup>a</sup>**

Component	Dry snuff			Moist snuff				
	A	B	C	A	B	C	D	E
Moisture (%)	4.7	5.6	5.4	45	20	49	50	51
Alkaloids								
Nicotine (mg/g)	11.9	14.7	11.7	13.8	4.6	7.4	10.4	15.0
Nornicotine (μg/g)	7.4	45.1	39.2	-	-	-	-	-
Anatabine (μg/g)	51.5	117	170	352	208	77	175	260
Anabasine (μg/g)	2.3	6.7	6.4	11	4.0	10	10	9.8
Myosmine (μg/g)	-	-	-	105	80	26	75	127
TSNA (μg/g)								
NNN	14.9	37.8	84.0	3.2	10.8	6.9	33.7	60.3
NAT	19.8	16.6	35.3	1.9	2.6	5.3	55.5	83.0
NAB	0.71	0.78	1.4	0.11	0.32	0.25	1.9	2.8
NNK	1.8	6.5	14.4	0.06	0.79	0.71	1.9	4.2
iso-NNAl	0.07	0.11	0.14	-	0.06	0.07	1.1	0.8
Total TSNA	37.3	61.8	135.2	5.3	14.6	13.2	94.1	151.1

<sup>a</sup>All values based on wet weights  
-, not determined

snuff dipping and chewing (Hoffmann & Adams, 1981; Palladino *et al.*, 1986), we tested NNN and NNK as carcinogens in the oral cavity of the rat.

The oral surfaces of rats were swabbed twice daily with an aqueous solution of NNN (135 ppm) and NNK (27.5 ppm). After two years of treatment of a group of 30 rats, eight animals had oral tumours and five had lung tumours (one adenoma and four adenocarcinomas; Table 2; Hecht *et al.*, 1986c). Swabbing of the oral cavity with a snuff extract enriched with NNN and NNK to the same level as that of NNN plus NNK alone resulted in fewer tumours of the oral cavity and lung. The genuine snuff extract by itself did not lead to tumours of the oral cavity or of the lung. These findings suggest that snuff contains agents that may inhibit its carcinogenicity, especially of the TSNA. Currently, we are exploring this aspect.

**Table 2. Tumour incidence in male Fischer 344 rats treated with NNN and NNK and with snuff extract by oral swabbing**

Group	Application ( $\mu\text{g}$ ) <sup>a</sup>		No. of rats	No. of rats with tumours	
	NNN	NNK		Oral cavity	Lung
NNN and NNK	68	14	30	8 <sup>b</sup>	5 <sup>c</sup>
Snuff extract enriched with NNN and NNK	74	15	30	3 <sup>d</sup>	2 <sup>e</sup>
Snuff extract	6.6	1.4	30	0	0
Control	-	-	21	0	1 <sup>e</sup>

<sup>a</sup>Twice daily for 120 weeks

<sup>b</sup>Six cheek papillomas, one hard-palate papillomas, two tongue papilloma

<sup>c</sup>One adenoma, four adenocarcinomas

<sup>d</sup>One cheek papilloma, one hard-palate papilloma, one tongue papilloma

<sup>e</sup>Adenoma

**Acknowledgements**

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# MUTAGENICITY AND CARCINOGENICITY OF *MASHERI*, A PYROLYSED TOBACCO PRODUCT, AND ITS CONTENT OF TOBACCO-SPECIFIC NITROSAMINES

S.V. Bhide<sup>1</sup>, J. Kulkarni<sup>1</sup>, U.J. Nair<sup>1</sup>, B. Spiegelhalder<sup>2</sup> & R. Preussmann<sup>2</sup>

<sup>1</sup>*Carcinogenesis Division, Cancer Research Institute, Tata Memorial Centre, Parel, Bombay 400 012, India; and* <sup>2</sup>*Division of Toxicology and Chemotherapy, German Cancer Research Centre, Heidelberg, Federal Republic of Germany*

*Masheri*, an indigenous pyrolysed tobacco product in India, was studied for its chemical, mutagenic and carcinogenic profile. *Masheri* extract was found to be rich in *N*-nitrosamines and polycyclic aromatic hydrocarbons. It was highly mutagenic in the presence of an exogenous metabolic system in the Ames test and in the micronucleus test, in a dose-dependent manner. It also induced 8-azaguanine-resistant mutants in Chinese hamster V79 cells. On skin painting, it showed a weak carcinogenic effect in Swiss nude mice. The saliva of *masheri* users showed high levels of *N*'-nitrosornicotine (NNN; 14-43 ppb) and *N*-nitrosopyrrolidine (NPYR; 2.2-8.3 ppb). Thus, this widespread habit, predominant among women, could be an additive risk factor in the high incidence of oropharyngeal cancer prevalent in India.

*Masheri*, or *mishri*, is a pyrolysed tobacco product used habitually in India as a substitute for chewing tobacco, predominantly by women, who usually place it between the gum and oral mucosa (Murdia *et al.*, 1982). Oropharyngeal cancer accounts for about 40% of all cancers in India, and the crude incidence rate in Bombay in 1983 was 15 per 100 000 population. In this paper, we present a chemical, mutagenic and carcinogenic profile of *masheri*.

## Determination of polycyclic aromatic hydrocarbons (PAH) in *masheri* extract

*Masheri* was extracted with toluene (Bhide *et al.*, 1984), enriched and analysed by gas chromatography (Grimmer & Bohnke, 1979). A wide spectrum of PAH, including established carcinogenic and cocarcinogenic compounds, was found to be present (Table 1).

## *N*-Nitrosamine content of *masheri* extract

In order to determine nitrosamines in *masheri*, 5 g were added to 40 ml distilled water and left at 40°C for 24 h, filtered and the residue washed with 20 ml water. The combined filtrates (pH 7.0) were extracted over Extreleut (E. Merck, Darmstadt, FRG) with dichloromethane; the eluate was then concentrated to 1 ml over a stream of nitrogen and analysed by gas chromatography-thermal energy analysis, as described by Spiegelhalder *et al.* (1979). High levels of *N*-nitrosodiethylamine (37 ppb), NPYR (31 ppb) and tobacco-specific nitrosamines (TSNA): *N*'-nitrosoanatabine (488 ppb), 4-(*N*-nitroso-methylamino)-1-(3-pyridyl)-1-butanone (NNK; 488 ppb) and NNN (932 ppb) were found in the *masheri* extract at pH 7.0.

**Table 1. Polycyclic aromatic hydrocarbon profile of *masheri***

Compound	Concentration (ng/g)
<i>Carcinogens</i>	
Benzo[ <i>a</i> ]pyrene	12
Benzo[ <i>fluoranthenes</i> [ <i>b,j,k</i> ]	35
Indeno[1,2,3- <i>cd</i> ]pyrene	6
Benzo[ <i>a</i> ]anthracene	79
Anthanthrene	11
Cyclopenta[ <i>cd</i> ]pyrene	6
<i>Weak carcinogens</i>	
Chrysene and triphenylene	192
Benzo[ <i>e</i> ]pyrene	17
Perylene	4
Coronene	5
<i>Cocarcinogens</i>	
Pyrene	169
Fluoranthene	218
Benzo[ <i>ghi</i> ]perylene	9
<i>Noncarcinogens</i>	
Benzo[ <i>ghi</i> ]fluoranthene and benzo[ <i>c</i> ]phenanthrene	66
<i>Other</i>	
Benzo[ <i>a</i> ]naphtho[2,1- <i>d</i> ]thiophene	57

A concentration of 50 µg/ml *masheri* extract (added for 24 h with an expression time of 7 days) increased the mutation frequency of 8-azaguanine-resistant mutants in Chinese hamster V79 cells.

### Carcinogenicity of *masheri*

The carcinogenicity of *masheri* was studied in nude Swiss mice (Cancer Research Institute) by applying an acetone solution of *masheri* extract (20 µl) to the midscapular region five times a week. Mice receiving 200 nmol 7,12-dimethylbenz[*a*]anthracene (DMBA) once and mice treated with acetone served as positive carcinogen and solvent controls, respectively; an untreated control group of the same age and strain was also available. The acetone-treated group showed mild hyperplasia. In the group treated with *masheri* extract, the incidence of papillomas (20%) was comparable to that induced by

### *N*-Nitrosamines in saliva of *masheri* users

Nine women aged 25-45 years were given 1 g of *masheri* and their saliva was collected over 20 min, extracted by the method of Bhide *et al.* (1986) and analysed for TSNA by gas chromatography-thermal energy analysis. NNN (14.3-40.5 ppb) was detected in all the samples; however, none of the samples contained NNNK. Relatively high concentrations of NPYR (2.2-8.3 ppm) were detected. Some samples also showed the presence of unidentified *N*-nitroso compounds (9.8-48.9 ppm).

### Mutagenicity of *masheri*

The mutagenicity of *masheri* was studied using one bacterial and two mammalian test systems. In the Ames test (Ames *et al.*, 1975), *masheri* extract induced a dose-dependent increase in the number of histidine revertants per plate in *Salmonella typhimurium* TA98 only in the presence of metabolic activation, indicating that it induces frameshift mutations.

For the micronucleus test (Schmid, 1975), two doses of 0.05 mg *masheri* extract were given intraperitoneally to Swiss male mice at an interval of 24 h, and the animals were killed 6 h after the second injection and polychromatic erythrocytes scored. The extract increased the levels of micronucleated erythrocytes in bone-marrow cells.

DMBA (18.4%); but the *masheri*-treated group showed lower incidences of papillomas with atypia (4.4%) and of malignant papillomas (2.2%) than the DMBA-treated group (18.4% and 10.4%, respectively).

### Conclusions

*Masheri* is rich in PAHs, some of which are known to be potent carcinogens, such as benzo[*a*]pyrene, DMBA and benzofluoranthenes. These, together with the high levels of both volatile and TSNA also present in *masheri*, could result in a potent combination. *Masheri* extract was highly mutagenic in the Ames test as well as in the two mammalian systems studied and was weakly carcinogenic at the dose level tested.

*Masheri* is used by a large section of the Indian population from once to as many as five to six times a day. It may thus be responsible for at least part of the high incidence of oral cancer in India.

# CORRELATION BETWEEN CHEWING AND SMOKING HABITS AND PRECANCEROUS LESIONS IN HILL TRIBES OF NORTHERN THAILAND

P. Reichart<sup>1,3</sup> & U. Mohr<sup>2</sup>

<sup>1</sup>*Abteilung für Zahnärztliche Chirurgie/Oralchirurgie,  
Polikliniken für Zahn-, Mund- und Kieferkrankheiten (Nord),  
Freie Universität Berlin, Föhrerstrasse 15, D-1000 Berlin (West); and*  
<sup>2</sup>*Institut für experimentelle Pathologie, Medizinische Hochschule Hannover,  
Hanover, Federal Republic of Germany*

In a field study, 1866 people from different hill tribes (Lahu, Karen, Lisu and Meo) of northern Thailand and a group of rural Thai were examined for chewing, smoking and drinking habits and precancerous lesions of the oral mucosa. Traditional chewing and smoking habits were more prevalent among older people; cigarette smoking, in contrast, was more prevalent in younger and middle-aged individuals. Preleukoplakia was observed in 1.8%, leukoplakia in 1.1% and chewer's mucosa in 13.1% of the study population. Men and older patients were affected more frequently; chewer's mucosa was seen more often in women (Karen, Thai). A correlation between precancerous lesions and some oral habits could be demonstrated statistically.

It has been suggested (Hoffmann *et al.*, 1984a) that the high concentrations of tobacco-specific *N*-nitroso compounds in tobacco preparations may be an important cause of the widely observed association between tobacco habits and oral cancer (IARC, 1985).

The high prevalence of oral precancer and cancer in India (Mehta *et al.*, 1971; Gupta *et al.*, 1980) and south-east Asia (Simarak *et al.*, 1977) has been associated with the use of tobacco and betel quid. The purpose of the present study was to correlate smoking, chewing and drinking habits of different hill tribe populations of northern Thailand with lesions of the oral mucosa. The study group consisted of 128 Lahu, 577 Karen, 139 Lisu, 506 white Meo and 516 rural Thai. For oral examination, the guidelines to epidemiology and diagnosis of oral mucosal diseases and conditions of the WHO were used (WHO, 1978).

## Chewing, smoking and drinking habits

The study population comprised 986 men and 880 women; 24.9% of all subjects were above the age of 40; 15.8% of the men and 18.9% of the women were betel-quid chewers. Tobacco was almost invariably used in the betel quid. The Meo did not chew betel; the highest prevalence of betel chewers was found among the Karen women (45.9%). *Miang* chewing was most prevalent among the Thai (40.5% of men, 43.7% of women). *Khi yo* cigars were smoked by 29.2% of the Thai men and 25.4% of the women; 22.7% of the Thai men, 23.4% Lisu, 6.9% Karen, 13.4% Meo and 9.1% Lahu were cigarette smokers. Cigarette smoking was statistically significantly more prevalent among men. Bamboo pipes were smoked exclusively by the Karen (37.0% of men, 45.9% of women). The habit of smoking opium was observed in all hill tribes except the Thai.

<sup>3</sup>To whom correspondence should be addressed



Among the Karen, both men (40.8%) and women (29.0%) drank alcoholic beverages. In the Thai and Meo, drinking of alcohol was quite prevalent among men (38.3% and 45.8%), but statistically significantly less so in the women.

### Prevalence of precancerous lesions

Preleukoplakia was observed in 1.8% of all subjects. Leukoplakia (n=21) was diagnosed in 1.1% of all persons; 1.5% of men and 0.7% of women were affected. Chewer's mucosa was found in 13.1% of all persons; the prevalence ranged from 38.1% in the Lahu to 3.0% in the Thai (men); chewer's mucosa was not found in the Meo. A statistically significant positive correlation was found between betel-quid chewing, *miang* use, age and chewer's mucosa. Due to the small number of persons with preleukoplakia and leukoplakia, only trends of correlations with betel-quid chewing and some smoking habits could be demonstrated.

### Future changes in habit patterns

The present study revealed considerable differences in chewing and smoking habits in different hill tribes of northern Thailand. The prevalence rates for betel-quid chewing were high in more primitive tribes (Lahu), but low in the Thai, among whom only older people indulged in this habit, indicating that the habit might disappear from Thai society. The habit of chewing *miang* is a typical Thai habit, adopted by some hill tribes; no study of the effects of *miang* chewing on the oral mucosa has so far been made. Tribe-specific smoking habits are still prevalent; however, younger people have started to replace these habits by cigarette smoking. Drinking is moderate in all tribes.

The prevalence of oral leukoplakia ranged from 0-3.2%. In India (Mehta *et al.*, 1971), the prevalence of oral leukoplakia ranged from 0.2-4.9% and was strongly correlated with the prevailing habits.

Chewer's mucosa was the lesion observed most frequently. It is considered to be an early form of submucous fibrosis, a probable precancerous lesion (Reichart *et al.*, 1984). Further longitudinal and morphological studies, as well as specific studies on the effect of *miang* chewing, may help to clarify the nature of precancerous lesions, especially chewer's mucosa.

## ENDOGENOUS NITROSATION IN THE ORAL CAVITY OF CHEWERS WHILE CHEWING BETEL QUID WITH OR WITHOUT TOBACCO

J. Nair<sup>1</sup>, U.J. Nair<sup>2</sup>, H. Ohshima<sup>1</sup>, S.V. Bhide<sup>2</sup> & H. Bartsch<sup>1</sup>

<sup>1</sup>Unit of Environmental Carcinogens and Host Factors, International Agency for Research on Cancer, 150 cours Albert-Thomas, 69372 Lyon, France; and <sup>2</sup>Carcinogenesis Division, Cancer Research Institute, Tata Memorial Centre, Parel, Bombay 400012, India

In order to evaluate endogenous nitrosation in the oral cavity of chewers of betel quid with tobacco (BQT) or without tobacco (BQ), saliva samples were collected from healthy male volunteers after chewing sequentially (i) unmodified BQT or BQ, (ii) BQT or BQ to which proline had been added, and (iii) BQT or BQ to which proline and ascorbic acid had been added. Samples were collected over 20 min and analysed for *N*-nitrosoproline (NPRO), tobacco-specific nitrosamines (TSNA) and areca nut-specific nitrosamines using gas chromatography-thermal energy analysis, arecoline and nicotine using gas chromatography-nitrogen phosphorus-specific detector, and for nitrite and thiocyanate. When results were expressed as a ratio of NPRO (ng/ml) to nicotine ( $\mu\text{g/ml}$ ), all BQT chewers had increased NPRO contents after chewing BQT with proline. For BQ chewers, when the results were expressed as a ratio of NPRO (ng/ml) to arecoline ( $\mu\text{g/ml}$ ), a similar increase in NPRO content was observed. However, the presence of ascorbic acid inhibited the increased nitrosation in only four out of ten BQT chewers and in five out of ten BQ chewers; in the rest of the samples, its presence enhanced the levels of NPRO. *N*-Nitrosoanatabine (NAT) and *N*-nitrosoguvacoline (NGCO) levels decreased significantly in saliva of chewers of BQT in the presence of ascorbic acid, suggesting inhibition of their formation. In-vitro nitrosation of BQT/BQ with proline and proline plus ascorbic acid showed a similar pattern of nitrosation at salivary pH. The study confirmed previous results that certain nitrosamines are formed during the chewing of BQT/BQ.

Carcinogenic *N*-nitroso compounds (NOC) have been detected in tobacco and in the saliva of chewers of tobacco and of BQT (Wenke *et al.*, 1984a; Hoffmann & Hecht, 1985; Nair *et al.*, 1985; Bhide *et al.*, 1986). In-vitro nitrosation of BQT and BQ has demonstrated that NOC can be formed at neutral pH (7.4, salivary) and acidic pH (2.1, gastric), leading to an increased exposure of chewers to NOC (Nair *et al.*, 1985). The present work was carried out to assess the formation of NOC *in vivo* in the oral cavity of chewers of BQT and BQ using the NPRO test (Ohshima & Bartsch, 1981), and to explore the use of ascorbic acid as an inhibitor of endogenous nitrosation.

### Study subjects, collection of samples and analysis of NOC and their precursors

Saliva samples were collected over 20 min from healthy male volunteers (ten subjects in each group) after chewing sequentially (i) unmodified BQT or BQ, (ii) BQT or BQ to which

100 mg proline had been added and (iii) BQT or BQ to which 100 mg proline and 100 mg ascorbic acid had been added. The samples were extracted and analysed for TSNA (*N*'-nitrosornicotine, NNN; NAT; 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone, NNK), areca nut-specific nitrosamines (NGCO; *N*-nitrosoguvacine, NGCI) and 3-(*N*-nitrosomethylamino)propionic acid (NMPA), 4-(*N*-nitrosomethylamino)butyric acid (NMBA) and NPRO by gas chromatography-thermal energy analysis and for arecoline and nicotine using capillary gas chromatography-nitrogen phosphorus-specific detection (Nair *et al.*, 1985). The samples were also analysed for thiocyanate (Pettigrew & Fell, 1972) and nitrite (Sen & Donaldson, 1978) colorimetrically.

For in-vitro nitrosation studies, extracts of BQT and BQ were prepared in 100 ml 0.2 M phosphate buffer (pH 7.4) according to a method reported earlier (Nair *et al.*, 1985). Twenty-ml aliquots were then treated as follows: one aliquot was used as a control without any treatment; to the second aliquot, nitrite (50 ppm) and thiocyanate (100 ppm) were added; to the third aliquot, nitrite and thiocyanate were added with 100 mg proline; and to the fourth aliquot, nitrite, thiocyanate and proline were added with 100 mg ascorbic acid. The pH was adjusted to 7.4 whenever necessary with dilute sodium hydroxide/hydrochloric acid, incubated at 37°C for 1 h and then extracted and analysed for NOC (Nair *et al.*, 1985).

#### pH, levels of NOC and their precursors in saliva

The pH, levels of NOC and their precursors in the saliva of chewers of BQT and BQ collected from the three groups as described above, are shown in Table 1. The pH of the samples obtained after chewing BQT or BQ with proline plus ascorbic acid was found to be lower than that in other groups. No significant difference in nitrite, thiocyanate, nicotine or arecoline levels was found between any of the groups. High concentrations (ng/ml) of carcinogenic TSNA (NNN and NNK) were found in all the samples collected from chewers of BQT. For the purpose of comparison of NOC in the three groups, the ratios of NOC to the precursor alkaloids were determined for each sample; the mean values are given in Table 2. NAT and NGCO values in the samples collected from BQT chewers with proline plus ascorbic acid were lower ( $p < 0.05$ ) than those in samples collected without those chemicals, showing inhibition of NOC formation. However, no such inhibition of NGCO was observed in the saliva of BQ chewers. No significant difference was observed for NNN levels, confirming the results of in-vitro studies (Nair *et al.*, 1985) that (i) most of the NNN in saliva originates from preformed compounds already present in tobacco and (ii) certain amounts of NAT and NGCO are formed in the oral cavity.

#### Formation of NPRO in the oral cavity of chewers of BQT and BQ

The formation of NOC in the oral cavity was further confirmed by the results of the NPRO test. When the data were expressed as a ratio of NPRO (ng/ml) to nicotine ( $\mu\text{g/ml}$ ) in saliva, all ten BQT chewers had an increased NPRO content after chewing BQT with proline (Fig. 1). For BQ chewers, when the results were expressed as a ratio of NPRO (ng/ml) to arecoline ( $\mu\text{g/ml}$ ) a similar increase in NPRO content was observed (Fig. 2). However, the presence of ascorbic acid inhibited the increase in nitrosation in only four out of ten BQT chewers and in five out of ten BQ chewers; in the rest of the samples its presence enhanced the levels of NPRO. The mechanism of the enhancing effect of ascorbic acid on NPRO formation under these experimental conditions remains to be elucidated, although ascorbate has been reported to catalyse nitrosation *in vitro* under certain conditions (Chang *et al.*, 1979). A number of simple phenolic and polyphenolic compounds are reported to be strong nitrosation modifiers (Pignatelli *et al.*, 1982), and tobacco and betel-quin constituents (areca nut and catechu) contain tannins and a large variety of polyphenols (e.g.,

**Table 1. Levels (range) of *N*-nitroso compounds and their precursors in saliva**

Parameter analysed	None	With proline	With proline plus ascorbic acid
<b>Betel quid with tobacco</b>			
pH	6.18-8.20	6.14-8.35	5.40-7.35
Nitrite ( $\mu\text{g/ml}$ )	0.4-40.5	0 - 31.9	0.2-76.0
Thiocyanate ( $\mu\text{g/ml}$ )	2.6-9.8	2.8-9.2	2.3-6.6
Arecoline ( $\mu\text{g/ml}$ )	1.9-70.0	3.0-67.9	8.9-91.2
Nicotine ( $\mu\text{g/ml}$ )	23.9-221.2	47.8-239.2	61.0-226.7
NNN (ng/ml)	4.9-48.6	11.3-49.0	11.6-42.5
NAT (ng/ml)	4.4-34.1	5.9-28.1	5.0-31.0
NNK (ng/ml)	TR - 9.4	TR - 7.1	TR - 8.7
NMPA (ng/ml)	2.6-41.2	1.7-23.0	1.4-20.6
NMBA (ng/ml)	0.5-82.3	TR - 36.4	0.4-31.0
GCO (ng/ml)	3.1-23.5	0.7-21.0	TR - 16.0
<b>Betel quid without tobacco</b>			
pH	6.51-7.60	7.00-7.94	5.35-7.80
Nitrite ( $\mu\text{g/ml}$ )	0.3-29.5	0.9-31.4	0.3-49.7
Thiocyanate ( $\mu\text{g/ml}$ )	2.2-8.1	3.0-7.1	1.4-8.4
Arecoline ( $\mu\text{g/ml}$ )	3.4-141.9	3.1-66.2	7.0-182.2
NGCO (ng/ml)	0.6-8.8	1.3-12.9	2.3-8.6

TR, trace

**Table 2. Effect of proline and proline plus ascorbic acid on salivary levels of TSNA and areca nut-specific nitrosamines in chewers of BQT and BQ**

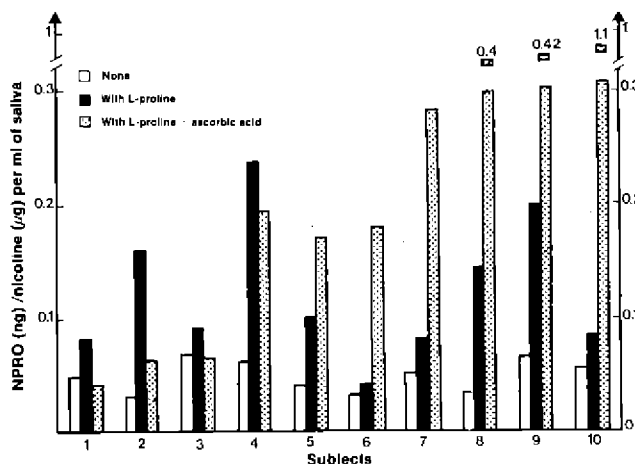
Sample	<i>N</i> -Nitrosamine (ng/ml)/ Alkaloid ( $\mu\text{g/ml}$ )	None	With proline	With proline plus ascorbic acid
BQT	NNN/nicotine	0.22±0.03	0.22±0.04	0.22±0.6
	NAT/nicotine	0.15±0.04	0.12±0.03	0.11±0.03*
	NNK/nicotine	0.02±0.02	0.01±0.02	0.02±0.03
	NMPA/nicotine	0.09±0.11	0.07±0.07	0.06±0.07
	NMBA/nicotine	0.13±0.20	0.07±0.10	0.06±0.01
	NGCO/arecoline	0.58±0.48	0.41±0.27	0.19±0.21*
BQ	NGCO/arecoline	0.15±0.06	0.22±0.12	0.18±0.11

\*Significantly lower when compared to sample collected without proline plus ascorbic acid (none),  $p < 0.05$

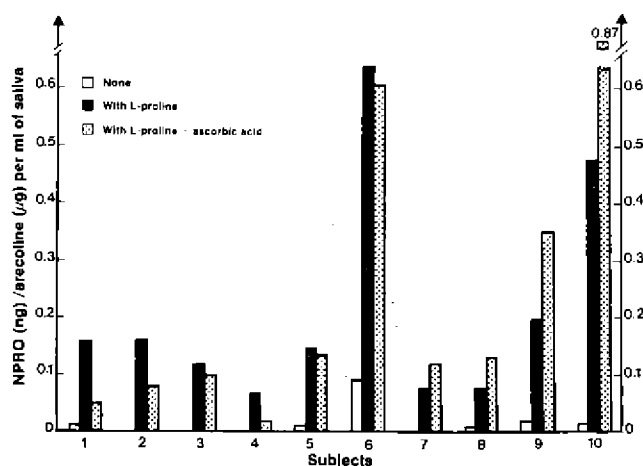
TR, trace

catechol, catechin, chlorogenic acid). Studies have shown that some of these polyphenols, such as catechin and areca-nut extracts, can both enhance and inhibit *N*-nitrosation *in vitro* in experimental animals and in humans (Pignatelli *et al.*, 1982; Stich *et al.*, 1983, 1984b). The effect depends strongly on pH, the nature of the polyphenolic compound involved and the ratio of the concentration of nitrite to polyphenol present in the reaction mixture.

**Fig. 1. NPRO levels formed in the saliva of chewers of BQT, while chewing an unmodified BQT, BQT to which 100 mg proline had been added or BQT to which 100 mg proline and 100 mg ascorbic acid had been added**



**Fig. 2. NPRO levels formed in the saliva of chewers of BQ, while chewing an unmodified BQ, BQ to which 100 mg proline had been added or BQ to which 100 mg proline and 100 mg ascorbic acid had been added**



### Correlation studies

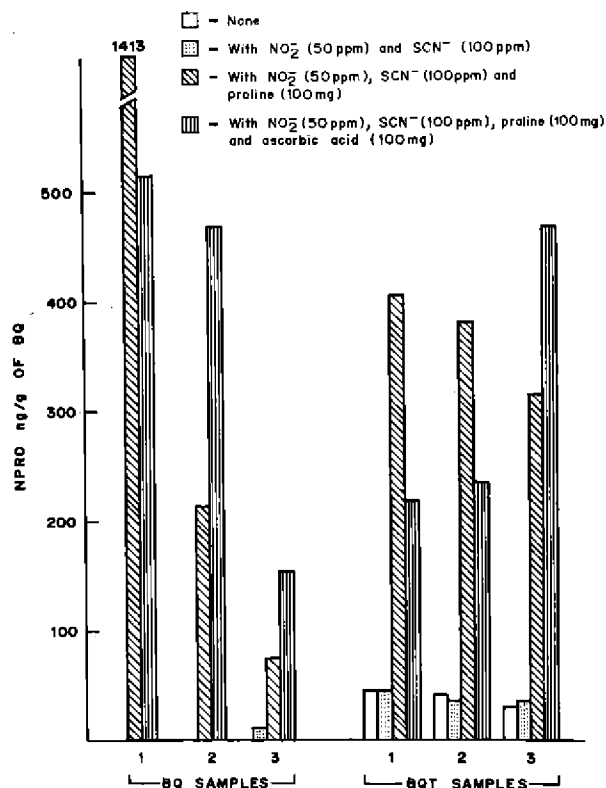
In order to establish the correlation between the different parameters studied in saliva, Spearman's rank correlation coefficients were calculated using the statistical package BMDP (W.J. Dixon, BMDP, Los Angeles, USA). Positive correlations were found between thiocyanate-nitrite, NMPA-NNN, NGCO-NAT, NMBA-NMPA ( $\alpha = 0.01$ ) and thiocyanate-NNN ( $\alpha = 0.05$ ) in the saliva. For the saliva of BQ chewers, a good correlation was found between thiocyanate-nitrite and NPRO-NGCO ( $\alpha = 0.01$ ). As reported in our earlier study (Nair *et al.*, 1985), no correlation was found between nitrite and any of the NOC.

### In-vitro nitrosation studies of BQT and BQ

In-vitro nitrosation studies of BQT and BQ containing proline and proline plus ascorbic acid showed the formation of NPRO in all the samples at pH 7.4 (Fig. 3). However, ascorbic acid was an effective inhibitor of nitrosation in only two of three BQT samples and one of three BQ samples, while in rest of the samples it enhanced NOC formation. NAT, NGCO and NGCI increased markedly

after incubation with nitrite and thiocyanate, and decreased after incubation with ascorbic acid. There was no significant difference in NNN, NNK and NMPA values (data not shown). The results are consistent with those of previous in-vitro nitrosation studies (Nair *et al.*, 1985).

**Fig. 3. NPRO levels in samples of BQ and BQT before and after in-vitro nitrosation and after nitrosation with proline and proline plus ascorbic acid**



**Conclusions**

Chewing of BQT is causally associated with human cancer (IARC, 1985), and it has been suggested that TSNA play a major role in the etiology of oral cancer (Hoffmann & Hecht, 1985; Nair *et al.*, 1985; Bhide *et al.*, 1986). The present study indicates that, apart from the preformed NOC present in BQT and BQ, a substantial fraction of TSNA and other NOC are synthesized *in vivo* in the oral cavity, thus increasing the body burden of NOC. It has been demonstrated that complete inhibition of endogenous nitrosation at salivary pH is not possible with the levels of ascorbic acid used (100 mg). Effective levels of ascorbate or any other nitrosation inhibitor may partly lower the body burden of NOC in BQT and BQ chewers; nevertheless, avoidance or cessation of betel chewing appears to be the only certain way to eliminate the cancer risk associated with these habits.

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## THE ROLE OF *N*-(NITROSOMETHYLAMINO)PROPIONITRILE IN BETEL-QUID CARCINOGENESIS

B. Prokopczyk, K.D. Brunnemann, P. Bertinato & D. Hoffmann

*Naylor Dana Institute for Disease Prevention, American Health Foundation,  
Valhalla, NY 10595, USA*

*N*-(Nitrosomethylamino)propionitrile (NMAP) was isolated and identified in the saliva of betel-quid chewers in amounts ranging from 0.5 to 11.4  $\mu\text{g/l}$ . Groups of 21 male and 21 female rats were given 60 subcutaneous injections of NMAP over a 20-week period (total doses, 0.055 and 0.23 mmol/rat). After 106 weeks, the higher dose had induced 18 (86%) malignant tumours of the nasal cavity in male and 15 (71%) in female rats. Nine (43%) liver tumours were observed among animals treated with the lower dose. Fischer 344 rats were treated with a single dose of NMAP (intravenously or subcutaneously, 0.4 mmol/kg; or by swabbing the oral cavity, 2.21 mmol/kg), and the levels of *N*7-methylguanine (7-meG) and *O*<sup>6</sup>-methylguanine (*O*<sup>6</sup>-meG) were measured in DNA isolated from oesophagus and nasal mucosa, which are target organs, and from liver which is not. Higher levels of *O*<sup>6</sup>-meG and 7-meG were detected in the nasal mucosa and lesser DNA methylation in the liver and oesophagus, independent of the mode of administration. This correlates with the results of the study of the tumorigenic properties of NMAP in rats.

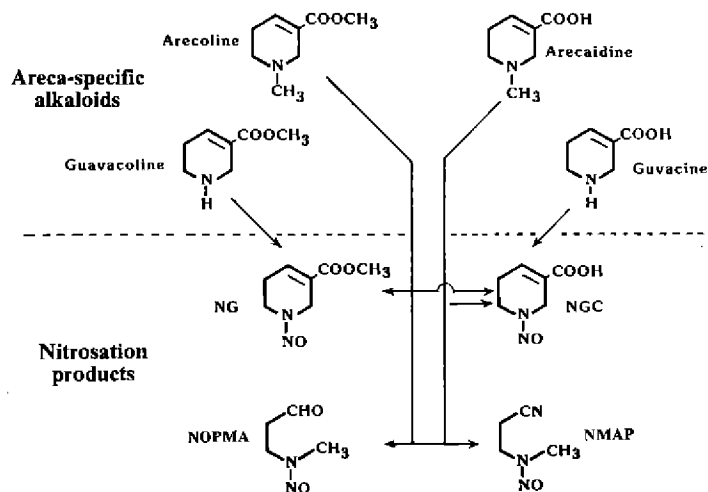
The significance of chewing of betel quid with and without tobacco in the etiology of oral cancer in India and other Asian countries has been well established (Hirayama, 1966; Jussawalla & Deshpande, 1971; IARC, 1985). Extracts of betel quid have induced tumours in laboratory animals, but specific carcinogens were not detected (Bhide *et al.*, 1979).

The known mechanisms of formation of nitrosamines from tobacco alkaloids (Hoffmann *et al.*, 1976) suggested that areca alkaloids might similarly be converted to nitrosamines. This hypothesis was confirmed in model studies on the nitrosation of arecoline (Wenke & Hoffmann, 1983; Fig. 1). Subsequently, the presence of the areca-derived nitrosamines, *N*-nitrosoguvacoline (NG) and *N*-nitrosoguvacine (NGC), was documented in the saliva of betel-quid chewers; however, NMAP and *N*-nitroso(3-oxopropyl)methylamine (NOPMA) were not detected (Wenke *et al.*, 1984b; Nair *et al.*, 1985). Preliminary results from bioassays of the synthesized areca-derived nitrosamines indicated that NMAP is a potent carcinogen in rats (Wenke *et al.*, 1984c). This presentation reports on further analytical investigations, on a second bioassay of NMAP in rats and on the alkylation of DNA in different tissues after administration of NMAP to rats by various routes.

### Isolation and identification of NMAP in the saliva of betel-quid chewers

Model studies suggested that NMAP in saliva is unstable. Therefore, betel chewers' saliva must be analysed with appropriate precautions and without delay (Prokopczyk *et al.*, 1987). For the identification of NMAP, we collected saliva samples (15-50 ml) during

Fig. 1 Nitrosation products of areca alkaloids



chewing of betel quid from Indians residing in the New York area. Immediately after collection, the samples were stored at 0-5°C in acetone:water. Volume and pH were determined, and each sample was continuously extracted with ethyl acetate; the organic layer was then dried and concentrated. The residue was subjected to column chromatography (silica gel, ethyl acetate) and was purified by preparative thin-layer chromatography (silica gel, diethyl ether:methanol, 9:1). The band corresponding to the R<sub>f</sub> of NMAP was extracted with methylene chloride and evaporated to dryness.

Each sample was then dissolved in ethyl acetate and analysed by gas chromatography-thermal energy analysis, using two different capillary columns (15 m × 0.25 mm Carbowax and 30 m × 0.25 mm Superox). Table 1 lists the levels of NMAP quantified in the saliva of betel-quid chewers. The identity of NMAP was confirmed by gas chromatography-mass spectrometry analyses (Fig. 2).

Table 1. NMAP content of saliva samples from chewers of betel quid and from controls

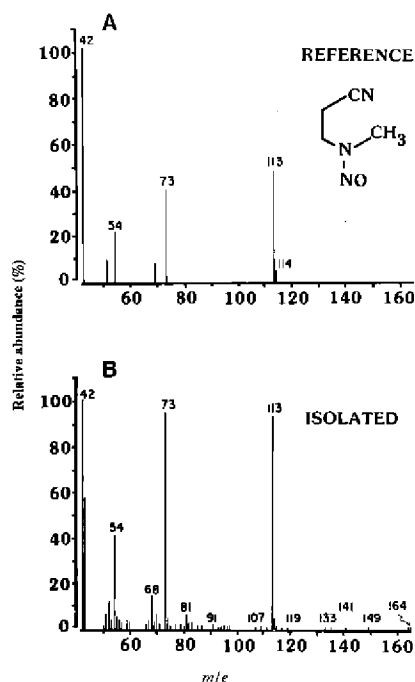
Specimen <sup>a</sup>	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Volume (ml)	22	15	30	32	26	35	38	50	48	36	32	30	35	40
pH	6.9	7.4	6.8	6.9	7.6	6.6	6.8	6.7	6.6	8.6	6.9	6.8	6.7	6.9
NMAP <sup>b</sup> (µg/l)	9.38	11.39	0.92	2.57	0.68	1.31	2.02	0.50	0.58	1.05	0.97	ND	ND	ND

<sup>a</sup>Specimens 1-10 were obtained from long-term chewers of tobacco-free betel quid, specimen 11 from a betel-quid chewer who is also a cigarette smoker and specimens 12-14 from nonchewers and nonsmokers.

<sup>b</sup>Isolated volumes  
 ND, not detected



**Fig. 2. Mass spectra of NMAP; A, reference, B, isolated from the saliva of betel-quin chewers**



### Tumorigenicity of NMAP in Fischer 344 rats

At the age of seven weeks, Fischer 344 rats of each sex were injected subcutaneously with either saline or NMAP (0.53 mg/kg or 2.13 mg/kg) in saline. Each experimental group (21 rats) was treated three times weekly for 20 weeks. The cumulative doses of NMAP per rat applied in 60 injections were 6.4 mg (0.055 mmol) and 25.7 mg (0.23 mmol), respectively. These doses correspond to 1/5 and 1/20 of the total dose given under the same conditions to Fischer 344 rats in an earlier study (Wenke *et al.*, 1984c). Vehicle controls comprised 12 male and 12 female rats.

The bioassay was terminated after 106 weeks. The tumour incidence, summarized in Table 2, demonstrates the organ-specificity of NMAP as a carcinogen for the nasal cavity (79%). All tumours were malignant tumours of the olfactory and respiratory mucosa, sometimes invading the brain or maxillary bone. The absence of nasal cavity tumours in the group treated with the lower dose may be due to the fact that the experiment was terminated too soon to show activity at that dose level; it was decided to terminate the experiment when the animals at the higher dose started to show weight loss and laboured breathing.

**Table 2. Induction of tumours in rats by subcutaneous injections of NMAP in saline three times weekly for 20 weeks**

Group	Sex	No. of rats	Number of rats with tumours				Bioassay terminated (weeks)
			Nasal cavity	Liver	Oesophagus	Tongue	
Controls	M	12	0	1	0	0	106
	F	12	1	3	0	0	106
NMAP, 0.055 mmol	M	21	1	9*	1	0	106
	F	21	0	0	0	0	106
NMAP, 0.23 mmol	M	21	18*	3	0	0	106
	F	21	15*	0	0	0	106
NMAP, 1.14 mmol <sup>d</sup>	M	15	11*	0	13*	5**	26
	F	15	9*	0	14*	6**	26

\*,  $p < 0.01$ ; \*\*,  $p < 0.05$

<sup>d</sup>Wenke *et al.* (1984c)

**Methylation of DNA by NMAP *in vivo* in Fischer 344 rats**

Previously described methods for DNA isolation and quantitative analysis of methylated purines were used (Herron & Shank, 1979; Hecht *et al.*, 1986a).

Upon intravenous or subcutaneous injection of a single dose of 0.4 mmol NMAP/kg to a male rat, we observed significant DNA methylation after 0.5-36 h, resulting in the formation of 7-meG and O<sup>6</sup>-meG (Table 3). The highest levels were observed in the nasal mucosa, with lesser DNA methylation in the liver and oesophagus. An identical pattern of methylation was observed after swabbing of the oral cavity with 2.21 mmol NMAP/kg. This indicates that, independent of the route of administration, NMAP is metabolically activated to a methylating agent in the nasal mucosa, liver and oesophagus, in agreement with the bioassay data.

**Table 3. Levels of 7-meG and O<sup>6</sup>-meG in Fischer 344 rat tissues measured after injection or swabbing of NMAP**

Treatment	Post-treatment interval (h)	Liver		Oesophagus		Nasal mucosa		Oral cavity	
		7-meG	O <sup>6</sup> -meG	7-meG	O <sup>6</sup> -meG	7-meG	O <sup>6</sup> -meG	7-meG	O <sup>6</sup> -meG
Intravenous	0.5	137	72	ND	ND	ND	ND		
	4	261	29	ND	ND	775	72		
	24	540	142	ND	ND	2260	156		
Subcutaneous	2	1169	44.7	150	ND	1301	82.7		
	6	2390	76.4	253	10.5	3716	291		
	24	1690	50.9	152	4.2	1505	169		
	36	1780	31.9	127	7.9	513	110		
Oral swabbing	24	1595	326	572	34.5	5900	587	127	23.2

ND, not detected

Comparative studies of DNA methylation clearly showed that levels of 7-meG and O<sup>6</sup>-meG formed in the liver upon treatment with *N*-nitrosodimethylamine greatly exceeded those formed with NMAP. However, liver tumour incidence was slightly higher with NMAP than with *N*-nitrosodimethylamine tested under similar conditions (Hecht *et al.*, 1986a). This finding indicates that factors other than the formation of these methylguanines may be involved in the tumorigenicity of NMAP.

In summary, this study has shown that NMAP, a potent areca-derived carcinogen, is present in the saliva of betel-quid chewers. NMAP exhibits organ-specific carcinogenic activity in rats by inducing tumours of the nasal cavity and, at high doses, also tumours of the oesophagus and of the tongue, NMAP is metabolically activated to a methylating agent in target tissues. However, factors other than formation of O<sup>6</sup>-meG may be involved in its tumorigenicity in Fischer 344 rats.

**Acknowledgement**

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## **CLINICAL AND EPIDEMIOLOGICAL STUDIES**

# N-NITROSO COMPOUNDS AS A CAUSE OF HUMAN CANCER

S. Preston-Martin

*Department of Preventive Medicine, University of Southern California,  
Los Angeles, CA 90033, USA*

Simultaneous consideration of epidemiological and experimental findings leads to the conclusion that the tobacco-specific nitrosamines (TSNA) 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK) and *N*'-nitrosonornicotine (NNN) probably cause oral and respiratory cancers in humans. The role of other *N*-nitroso compounds (NOC) in the etiology of human cancer may best be defined by the study of known human carcinogens, such as certain foods eaten exclusively by populations with exceptionally high incidences and mortality rates for certain cancers. The evidence that NOC may be responsible for these elevated rates is reviewed in relation to two such high-risk populations: (i) residents of Lin-xian county in northern China, who have a striking excess of cancer of the oesophagus, and (ii) Cantonese people who have high rates of nasopharyngeal carcinoma (NPC). More studies are needed to define clearly the relationship between various types of cancers and prenatal or lifetime exposures to passive smoking. If passive smoking is found to increase the risk of developing various childhood and adult cancers, the NOC in tobacco are likely to be among the relevant carcinogens. Carcinogenesis models for studying the effects of prenatal exposure to NNK and NNN and other NOC seem particularly promising for the study of low-dose effects and modifying factors.

Investigators have yet to show conclusively that a specific NOC causes human cancer. This link may best be established by research focusing on known human carcinogens that contain NOC.

## Personal use of tobacco products

Most substances known to be carcinogenic to humans are a complex mixture of compounds; often, several of them cause cancer in laboratory animals. NOC are probably among the active compounds in the carcinogenic mixtures used by humans that are listed on Table 1. All of these mixtures are tobacco products, which have been shown to contain several TSNA, including high levels of NNK and NNN (Hoffmann *et al.*, 1984a). Both NNK and NNN have been conclusively established as carcinogenic to laboratory animals (IARC, 1985). When NNK and NNN are administered experimentally in food or by subcutaneous or intraperitoneal injection, they cause cancers predominantly in the nasal cavity, trachea and lung (Table 2). In contrast, people who use the NNK- and NNN-containing tobacco products listed in Table 1 either retain them against their gum or buccal mucosa for prolonged periods (as with snuff), keep them in their mouths and chew them (*khaini* and betel quid) or draw the smoke from smouldering tobacco through the mouth, oropharynx and hypopharynx, past the opening of the oesophagus and through the larynx into the lung. These tobacco habits have been shown to cause cancer at all these body sites (Table 1). Thus, it seems likely that the NNK and NNN in tobacco products cause oral and respiratory

cancers in humans. Experiments with NNK and NNN using routes of administration that are similar to the human practices of snuff dipping and tobacco chewing and smoking would strengthen existing evidence for an etiological role of these NOC in human cancers. In one such study, tumours were produced in tissue in the oral cavity of rats swabbed repeatedly with a solution containing NNK and NNN (Hecht *et al.*, 1986c).

**Table 1. Some specific exposures that cause cancer in humans<sup>a</sup>: N-nitroso compounds probably among the active carcinogens**

Exposure	Cancer site
Snuff — oral use	Buccal mucosa; gum
Tobacco and lime — oral use	Oral cavity
Chewing betel quid with tobacco	Oral cavity
Smoking cigarettes	Lung; larynx; oral cavity; bladder; renal pelvis; pancreas
Smoking tobacco (cigarettes, cigars, pipes, <i>bidis</i> )	Oral cavity; oropharynx; hypopharynx; larynx; oesophagus

IARC (1985, 1986)

**Table 2. Carcinogenic effects of two TSNA that cause cancer in animals<sup>a</sup>**

TSNA	Route of administration	Experimental animal	Cancer site
NNK	Subcutaneous injection	Rat	Nasal cavity, lung, liver
NNK	Intraperitoneal injection	Hamster	Nasal cavity, trachea, lung
NNN	Oral	Rat	Oesophagus, nasal cavity
NNN	Subcutaneous injection	Hamster	Nasal cavity
		Rat	Nasal cavity
NNN	Intraperitoneal injection	Hamster	Trachea
		Hamster Mouse	Nasal cavity Lung

<sup>a</sup>IARC (1985)

Biochemical studies with laboratory animals have shown that TSNA are metabolically activated to  $\alpha$ -hydroxynitrosamines. These metabolites are unstable and decompose to highly reactive diazohydroxides, which function as alkylating agents. When animals are injected with NNK, *O*<sup>6</sup>-methylguanine and *O*<sup>4</sup>-methylthymidine are formed in the DNA of lung, liver and nasal mucosa (Hoffmann & Hecht, 1985; Belinsky *et al.*, 1986a); these are organs in which NNK induces tumours. Studies with cultured human buccal mucosa, trachea, oesophagus, bronchus, peripheral lung and bladder have shown that these tissues too can metabolize NNK by  $\alpha$ -hydroxylation (Castonguay *et al.*, 1983a). Therefore, it seems reasonable to expect that chewing of tobacco and smoking lead to formation of methylated bases in the DNA of oral or bronchial tissues of tobacco users. *O*<sup>6</sup>-Methylguanine and *O*<sup>4</sup>-methylthymidine cause mis-coding of DNA and may, therefore, be important in tumour initiation.

### Studies of high-risk populations

Recent findings in populations with exceptionally high incidence rates of oesophageal cancer or NPC suggest that studies of high-risk groups may help establish a definitive link between a specific NOC and human cancer. Exposure to NOC of populations at high risk of gastric cancer is discussed in this volume by P. Correa and will not, therefore, be discussed in this paper. The high levels of NOC in the urine of Egyptian patients with schistosomiasis may relate to their high incidence of bladder cancer (El-Merzabani *et al.*, 1979), but constraints on the length of this paper preclude discussion of that high-risk population.

The annual age-adjusted mortality rates for oesophageal cancer among people in Lin-xian county of northern China are 151/100 000 in males and 115/100 000 in females; rates among men and women in Fan-xian county, located 150 km east, are four to seven times lower (Office of Prevention and Treatment of Cancer, 1978). Dietary surveys have shown a positive correlation between the mortality rate from oesophageal cancer and the amount and frequency of consumption of pickled vegetables (Co-ordinating Group for Research on the Etiology of Esophageal Cancer in North China, 1977), and Chinese pickled vegetables have been shown to contain a recently identified nitroso compound, Roussin's red methyl ester (Wang *et al.*, 1980). These surveys also indicated that the levels of nitrate and nitrite in drinking-water, foods and saliva are higher in Lin-xian than in Fan-xian county. Low levels (ppb) of various NOC were detected in all of a variety of food samples, including millet flour, turnip chips, steamed corn cakes, steamed wheat cakes and persimmon bran meal, from houses in the Lin-xian area (Singer, G.M. *et al.*, 1986). Relatively high levels of NOC precursors (nitrite, nitrate and secondary amines) were found in foods, including wheat, corn, millet and pickled vegetables, eaten in Lin-xian (Co-ordinating Group for Research on the Etiology of Esophageal Cancer in North China, 1975). NOC are formed in Chinese corn bread after fungal contamination (Li *et al.*, 1979), and two of the amines in the corn bread, when fed together with nitrite, can cause cancer of the oesophagus and forestomach in rats (Lu & Lin, 1982); it should be remembered, however, that corn bread and pickled vegetables are eaten throughout China, although the methods of preparation vary by region. Compared to residents in Fan-xian, residents of Lin-xian had significantly higher urinary levels of three NOC (*N*-nitrosoproline, *N*-nitrosothiazolidine 4-carboxylic acid and *N*-nitrososarcosine) and of nitrate (Lu *et al.*, 1986). This finding indicates a higher exposure of Lin-xian residents to NOC and their precursors. Data from these several studies also suggest that the endogenous formation of NOC is higher among residents of Lin-xian. Exposure to NOC can cause elevated levels of *O*<sup>6</sup>-methyldeoxyguanosine in DNA, and such levels have been found in the DNA of oesophageal and stomach mucosa from cancer patients in Lin-xian (Umbenhauer *et al.*, 1985).

Lin-xian residents eat little fresh fruit and vegetables (Lu & Lin, 1982) and have lower blood levels of vitamin C and other vitamins than do residents of low-risk areas (Yang *et al.*, 1982). Intake of 100 mg ascorbic acid 1 h after each meal reduced urinary levels of NOC in Lin-xian residents to those found in residents in the low-risk area (Lu *et al.*, 1986). Intervention studies using vitamin C and other vitamins and minerals in Lin-xian may prevent the development of oesophageal cancer and lower mortality rates; these studies may also provide additional support for the hypothesis that NOC cause the excess of oesophageal cancer (Blot & Li, 1985).

The annual age-standardized incidence rate of NPC in males in central Guangdong in southern China is over 30/100 000 (Yu *et al.*, 1981). This rate is many times higher than those in other parts of China and in most other countries, where rates are less than 1/100 000 (Waterhouse *et al.*, 1982). The work of M. Yu and her colleagues has established salted fish

as a cause of NPC in Cantonese populations now living in various geographic areas (Yu *et al.*, 1986). Although that work showed that over 90% of NPC cases among Hong Kong Chinese under the age of 35 were attributable to intake of salted fish during childhood, this finding does not eliminate the Epstein-Barr virus (EBV) as a possible causative agent. It appears, however, that EBV infection is not sufficient to cause NPC, since there is a lack of correlation between the geographical and racial distributions of EBV infection and NPC incidence rates (Yu *et al.*, 1986).

Although NPC excesses have been attributed to a single food, the evidence available to date that the cancer excess is related to NOC seems stronger for oesophageal cancer in Lin-xian than for NPC among the Cantonese. Rats fed Cantonese-style salted fish have developed carcinomas of the nasal and paranasal regions (Huang *et al.*, 1978b; Yu & Henderson, this volume). Some NOC which cause cancers of the nasal and paranasal cavities in rodents are present at low levels (ppb) in Cantonese-style salted fish (IARC, 1978; Huang *et al.*, 1981), but the same preformed NOC are present at similarly low levels in cured meats eaten in Europe, where the incidence of NPC is very low. Fish samples are presently being analysed in a systematic search for the carcinogenic substance or substances or their precursors that cause the excess of NPC. Hopefully, this search will indicate whether or not NOC are a major factor in the induction of this cancer. If it seems that they are, analyses of biological samples from high-risk individuals may also be indicated (see discussion of Lin-xian region above).

#### **Exposure to other people's cigarette smoke (passive smoking)**

The levels of volatile NOC and of TSNA are far higher in undiluted sidestream than in mainstream cigarette smoke, and the uptake of sidestream smoke can be substantial for nonsmokers as well as for smokers (Hoffmann *et al.*, 1984b). This exposure may be particularly high in infants whose mothers smoke (Greenberg *et al.*, 1984). Studies that suggest that exposure to smoke from other people's cigarettes increases cancer risk are, therefore, important to consider here.

Results from early epidemiological studies of lung cancer in nonsmokers were conflicting, but, now that findings from more such studies are available, an etiological role for passive smoking is suggested (IARC, 1986). In fact, considering the relatively small increases in risk observed and the diversity of the studies with respect to design, methods and populations studied, the findings are remarkably consistent.

How do the risks of cancers other than lung cancer in both adults and children relate to prenatal or to lifetime exposures to passive smoking? These associations have not been widely studied, but papers reporting such associations are starting to accumulate (Hirayama, 1984; Sandler *et al.*, 1985). Hirayama's findings of a dose-related effect on cancer of the nose in nonsmoking women [relative risks (RR), 1.0, 1.7, 2.0 and 2.6 in women whose husbands smoked 0, 1-14, 15-19 and 20+ cigarettes per day, respectively], and his finding of increasing risks of lung cancer in these women, are consistent with studies that have related lung cancer and nasal cancer to personal cigarette smoking (Brinton *et al.*, 1984; IARC, 1986). However, the association of nasal cancer with personal smoking is not solidly established and must be confirmed in future studies; the finding that associates passive smoking with nasal cancer and a similar finding for brain cancer also await support or refutation. Experimental and epidemiological findings do suggest that cancers of the nasal cavities (see above) and brain tumours (Preston-Martin & Henderson, 1984) may be related to exposure to NOC, but experimental data indicate that nitrosamides rather than nitrosamines are the NOC that cause brain tumours.

An important issue is the possible increase in cancer risk from exposure to parents' smoking during early life. Both mother's and father's smoking were associated with risk of haematopoietic cancers diagnosed at ages 15 to 59 years, and the RR increased from 1.0 to 1.7 to 4.6 when neither, one or both parents smoked (Sandler *et al.*, 1985). In that study, the haematopoietic tissue was the only cancer site for which statistically significant elevations in risk were seen in association with both mother's and father's smoking. A recent study of maternal smoking during pregnancy and risk of childhood cancer supports these findings (Stjernfeldt *et al.*, 1986). The relative risks of acute lymphoblastic leukaemia in children under age 17 (132 cases) were 1.0, 1.3 and 2.1 if the mother smoked 0, 1-9 and 10 or more cigarettes per day, respectively. Interestingly, several mothers had stopped smoking when they became pregnant, and in fact, the strongest association is with smoking before pregnancy; however, these mothers were likely to have smoked during the early weeks of pregnancy. One is struck by the fact that in this study the increases in risk related to increased levels of daily cigarette consumption occur at quite low overall consumption levels, with a highest exposure category of 10 or more cigarettes per day. Two early studies of childhood leukaemia did not show statistically significant associations but did find slightly elevated risks (1.1 and 1.3) related to the mother ever smoking (Stewart *et al.*, 1958; Neutel & Buck, 1971). Two other studies showed no association (RR, 1.0) (Manning & Carroll, 1957; Van Steensel-Moll *et al.*, 1985). Unfortunately, details of smoking habits are not available in some of the early studies, and, even in the recent studies, the data collected are often not directly comparable. Comparable data from additional studies are needed before the association of leukaemia with parental smoking can adequately be evaluated.

### Prenatal exposures to NOC

Experimental models of NOC carcinogenesis are most fully developed for rodents. *N*-Ethyl-*N*-nitrosourea is a directly-acting NOC which induces neurogenic tumours (of the brain, meninges, spinal cord and nerves) in a variety of species, including rats, mice and rabbits. In rodents, susceptibility to induction of these tumours is greatest during late gestation and during the early postnatal period, and fetuses are 10-50 times more sensitive to the effects of this compound than are adult animals (Rice & Ward, 1982). *N*-Ethyl-*N*-nitrosourea also causes neurogenic tumours in patas monkeys, but in the monkey the period of greatest susceptibility is early gestation; no central nervous system tumour is produced by postnatal exposure (Rice & Ward, 1982).

Neurogenic tumours account for only about 1.5% of all cancers in adults but are the most common solid tumours in children (Waterhouse *et al.*, 1982). Transplacental carcinogenesis studies in nonhuman primates, such as those in progress at the NCI Frederick Cancer Research Facility (Table 3), seem particularly likely to add to our understanding of the importance of NOC and other carcinogens in the etiology of childhood brain tumours. Prenatal exposure models seem especially promising for studying the effects of relatively low-dose exposures to NOC and of modifying factors, both because fetuses are likely to be more sensitive than adults to the carcinogenic effects of NOC and because the exposure period of greatest interest (pregnancy) is clearly defined.

The hypothesis that prenatal exposure to NOC might relate to the development of childhood brain tumours was suggested by one epidemiological study (Preston-Martin *et al.*, 1982). When we undertook that case-control study in Los Angeles, California, USA, in the mid-1970s, we recognized that it had several major limitations. Analysis of NOC contamination in human environments and consumer products had been limited to nitrosamines, since assays to detect nitrosamides (including nitrosoureas) in such samples



**Table 3. Some objectives of transplacental carcinogenesis studies with *N*-nitroso compounds in monkeys<sup>a</sup>**

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To characterize susceptibility to chemical carcinogens
To identify the period of greatest susceptibility
To determine if characteristic human paediatric tumours (e.g., of the brain) can be induced
To determine if primate fetuses (like rodent fetuses) are more susceptible than adults
To determine whether characteristic human adult tumours can be induced
To explore the effects of potential promoters

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<sup>a</sup>Work in progress of A.E. Palmer and J.M. Rice at the Laboratory of Comparative Carcinogenesis, NCI Frederick Cancer Research Facility, Frederick, MD, USA

were not yet available. We were unable, therefore, to collect information on exposure to nitrosoureas, and we were uncertain of the relevance of the nitrosamine exposures we asked about in interviews with mothers of cases and controls. Most exposures, such as those to cosmetics and in particular occupational settings, were complex and were, therefore, not specific in that they involved exposure to a variety of other chemicals besides NOC. For most exposures, we had only crude estimates of dose. In that first study, we did not attempt to distinguish possibly different effects of the same exposure during various time periods. We were concerned about the possibility that recall bias might occur, because mothers of brain tumour patients might try harder to remember exposures, in particular to variables such as pesticides which they might already think of as 'bad'. Since about half of the exposure to NOC of nonsmokers was estimated to be from compounds formed in the stomach after simultaneous ingestion of foods and drugs that contain NOC precursors, we also asked mothers about drug use during the index pregnancy and about intake of certain foods. We decided, however, not to obtain a complete dietary history and, therefore, were unable to measure total intake of dietary constituents of interest. We also lacked information on the timing of intake of various precursors, catalysts and inhibitors. Further, we asked about only some of many experiences that may involve exposure to NOC or NOC precursors.

In that case-control study of brain tumour patients under age 25 years (209 matched pairs) in Los Angeles County, increased risk was associated with maternal contact during the index pregnancy with *N*-nitrosamine-containing substances such as burning incense (RR, 3.3; one-sided  $p < 0.01$ ), sidestream cigarette smoke (RR, 1.5;  $p = 0.03$ ) and face make-up (RR, 1.6;  $p = 0.02$ ). Increased risk was also associated with maternal use of diuretics (RR, 2.0;  $p = 0.03$ ) and antihistamines (RR, 3.4;  $p < 0.01$ ) and with the level of maternal consumption of cured meats ( $p < 0.01$ ). Diuretics and antihistamines contain nitrosatable amines and amides, and cured meats contain nitrites — chemicals that are precursors of NOC. Although most of the nitrite in the human stomach (where most of the endogenous formation of NOC appears to occur) comes from nitrate in vegetables, we did not find an association between consumption of high-nitrate vegetables and brain tumours. This lack of association may be explained by the fact that vegetables also contain vitamins and polyphenols, which are effective inhibitors of nitrosation. Our data did suggest that consumption of citrus fruit (which contains high levels of vitamin C) and prenatal vitamins

may protect against brain tumour development. We proposed a hypothesis that brain tumours in these young people are related to exposure to NOC and their precursors *in utero* and, furthermore, that maternal consumption of vitamins and of foods such as fresh fruits and vegetables which contain nitrosation inhibitors protects against brain tumour formation.

In that study, risk in patients diagnosed under age 10 (92 case-control pairs) was also associated with parental occupational exposures, including mothers' exposure to chemicals (RR, 2.8;  $p = 0.03$ ); fathers' exposure to solvents (RR, 2.8;  $p = 0.02$ ), in particular to paints (RR, 7.0;  $p = 0.04$ ); and fathers' employment in the aircraft industry (RR,  $\infty$ ;  $p = 0.001$ ; Peters *et al.*, 1981). Although the levels of exposure to NOC are known to be high for workers in some occupational settings, NOC exposures in aircraft factories have not been determined (National Research Council, 1981).

In the USA, the major sources of exposure to NOC for the general population include tobacco smoke, cosmetics and cured meats (National Research Council, 1981). NOC levels in tobacco smoke and cured meats have been widely studied, but further investigation of the levels of NOC (including levels of various nitrosamides and of *N*-nitrosodiethanolamine) in cosmetics is warranted, particularly in light of recent studies in which *N*-nitrosodiethanolamine was found to be carcinogenic to rats at quite low concentrations (Lijinsky & Kovatch, 1985). Perhaps further experimental studies are also needed to determine whether carcinogenic NOC might be formed after ingestion of various diuretic drugs. Drugs in this group are commonly prescribed for pregnant women, and another case-control study of neurogenic tumours in children showed recently that maternal use of these drugs during pregnancy increases the child's risk of tumour development (Kramer *et al.*, 1987). That study of 104 young children with neuroblastomas found a RR of 5.8 (90% confidence interval, 1.9-23.0) associated with maternal use of diuretics. Both the descriptive epidemiology and the histology of neuroblastomas are distinct from those of the various types of brain tumours, however, and the etiology of these two groups of tumours is likely to be different as well.

The hypothesis that NOC exposure is related to childhood brain tumours is currently under investigation in an international collaborative case-control study of these tumours now being coordinated by the IARC. This large study was designed to avoid some of the limitations of the Los Angeles study but will still face several of the methodological difficulties (discussed above) which are inherent in epidemiological studies of NOC and cancer. Both mothers and fathers of brain tumour patients will be interviewed. Sections of the questionnaire that refer to potential exposures to NOC, to NOC precursors and to modulators of nitrosation reactions are listed in Table 4. Readers of this paper are invited to comment on this list.

### Conclusions

The evidence that specific NOC cause cancer in humans is strongest for NNK and NNN in snuff; evidence implicating TSNA in tobacco smoke is less strong. No specific NOC has as yet been suggested to be responsible for the cancer excess observed in any of the high-risk populations discussed in this paper. Alternative etiological hypotheses (relating to substances other than NOC) cannot be excluded when considering cancer excesses in these high-risk groups and in tobacco smokers. Continued investigation of NOC as possible carcinogenic agents is, however, clearly warranted.

**Table 4. Interview questions about NOC from the international collaborative study of childhood brain tumours coordinated by the IARC, 1985-1990**

Questions asked about	Potential source of NOC exposure queried
Both parents	Job history History of tobacco use and passive smoking
Mother	Use of cosmetics
Mother and child	Household water supply Time spent in new cars Diet history Drug history Vitamin use
Child	Use of bottle or pacifier (dummy)

The most promising route to defining the role of specific NOC in the etiology of human cancers may be the epidemiological and experimental investigation of known human carcinogens, such as tobacco smoke and certain foods, including Lin-xian pickled vegetables and Cantonese-style salted fish. Cancers of primary interest are of the stomach, oesophagus, oral cavity, bladder and others which occur in excess in highrisk populations. Studies of prenatal exposure to NOC in nonhuman primates may help bridge the gap between

experimental models developed with rodents and as yet poorly defined associations of childhood tumours with prenatal exposures. Investigators of cancers in children and adults are encouraged to ascertain parents' smoking histories. Experimental studies of prenatal exposure to tobacco smoke or to NNN and NNK also seem particularly pertinent. For all of these human situations, it is not sufficient to study the presence or absence of exposure to a particular NOC; rather, details of the exposure, including various factors that potentiate the carcinogenicity of NOC (e.g., age at exposure, timing of exposure in relation to other exposures, relative absence of inhibitors, dose), must also be investigated.

Primary preventive measures must not await conclusive epidemiological results. Opportunities should be seized now to reduce further the levels of NOC, NOC precursors and the potential for nitrosation from human exposures to rubber products, foods, including processed meats, fish, vegetables and beer, drugs, cosmetics and tobacco.

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## **MODULATION OF GASTRIC CARCINOGENESIS: UPDATED MODEL BASED ON INTRAGASTRIC NITROSATION**

**P. Correa**

*Department of Pathology, Louisiana State University Medical Center,  
1901 Perdido Street, New Orleans, LA 70112, USA*

**The etiological model of gastric carcinogenesis is discussed. Its complexity and flexibility may be more applicable to human situations than experimental models utilizing high doses of carcinogens in homogeneous animal populations. The limitations of the epidemiological method and the use of a collaborative approach by epidemiologists and experimentalists are described. The need for markers of exposure to *N*-nitroso compounds is stressed.**

The main objective of the work reported at this conference is to test the hypothesis which links *N*-nitroso compounds to human cancer causation. This link is explored in both laboratory studies and in human populations. There is a need for mutual understanding and an opportunity for fertile collaboration between the two styles of scientific approach.

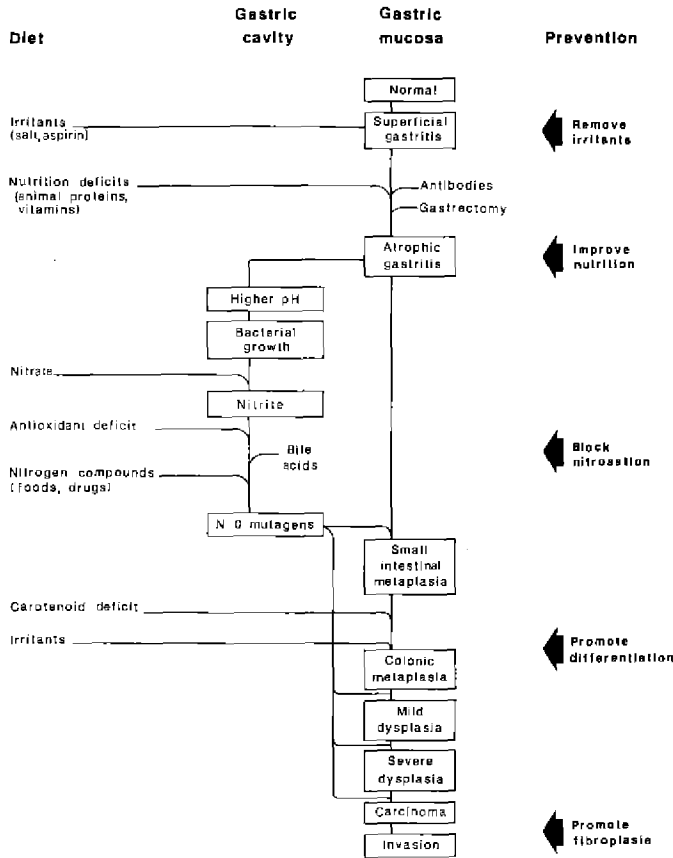
Epidemiology is mostly an observational science which aims to predict future human events on the basis of past events. It should not be expected to provide absolute scientific (experimental) proof of causation. Experimental sciences applied to nonhuman systems can be used to prove causation in such systems, but, again, there are problems in extrapolating such findings to human subjects. The main contribution of epidemiology to experimental sciences, therefore, is to signal the relevance of specific experiments to human events (past, present or future). Harmonious interdisciplinary work keeps expectations in line with the ability to deliver scientific guidance, especially when the answers sought concern human subjects. To illustrate collaborative efforts between the epidemiological and laboratory approaches, we examine the model of gastric carcinogenesis.

### **Gastric carcinogenesis model**

From direct observations in human populations, the distribution of gastric cancer has been described. It is determined primarily by cultural characteristics, which point to diet as the major determinant of risk. Gastric cancer incidence in humans correlates positively with the prevalence of chronic atrophic gastritis (CAG), which has emerged as the key precursor event in human gastric carcinogenesis. CAG alters the gastric microenvironment, increasing the pH and facilitating bacterial growth. As is the case for other inflammatory conditions, gastritis may increase endogenous nitrate synthesis (Stuehr & Marletta, this volume). CAG in high-risk populations is characteristically multifocal and frequently followed by a series of cell transformations, usually interpreted as successive mutations. The first and most prevalent transformation results in intestinal metaplasia. While in most patients intestinal metaplasia is well-differentiated, the cellular phenotype in a few individuals becomes progressively dysplastic and may finally become neoplastic. These apparently progressive stages have been documented in several populations and form the backbone of the

etiological model outlined in Figure 1. Most investigators interpret the morphological evolution as progressive loss of cellular differentiation, probably related to successive mutations (Correa, 1983).

**Fig. 1. Hypothesis for the chain of causation of gastric neoplasia**



Correlations of disease frequency with diet, as well as studies of patients with and without the disease (case-control), have pointed to dietary factors as etiological agents (Table 1; Fig. 1; Correa *et al.*, 1985). These agents can be grouped as follows:

**Irritants**

Irritants are factors that are directly capable of damaging the gastric mucosa. The most prominent of these is table salt, the intake of which is excessive in all human populations at high risk for gastric cancer (Joossens & Geboers, 1981). Salt has also been shown to increase the risk of gastric cancer and of chronic gastritis in analytical epidemiological studies

**Table 1. Factors implicated in gastric carcinogenesis**

Factor	Action	Timing
<b>Irritants</b>		
Salt	Osmotic damage?	Pre-initiation promotion?
	Mitogenic	Facilitation of carcinogen action
	Induction of oxidative decarboxylation	
Alcohol	?	?
Tobacco	?	?
Aspirin	?	?
<b>Atrophy inducers</b>		
Parietal-cell antibodies	pH elevation allows bacterial growth	Pre-initiation
Starchy and salty diets	Hormonal milieu?	Promotion
Mutagens - N-N=O	Genotoxicity	Initiation
<b>Antioxidants</b>		
Vitamin C	Block nitrosation	Initiation
Vitamin E		
Carotenoids	Scavenge free radicals Promote differentiation	Promotion
<b>Genetics</b>		
Autosomal recessive gene	Susceptibility to gastritis	Pre-initiation

(Fontham *et al.* 1986). Experimentally, salt induces gastritis by directly damaging the mucosa, increasing the mitotic rate (Charnley & Tannenbaum, 1985); and it somehow increases the effectiveness of carcinogens when given concurrently (Shirai *et al.*, 1982). Alcohol, tobacco and aspirin are also ubiquitous irritants associated with increased cancer risk (Hirayama, 1981; Hoey *et al.*, 1981; Correa *et al.*, 1985) and with enhancement of experimental carcinogenesis (Tsung-Hsieu *et al.*, 1983).

#### *Inducers of gastric atrophy*

The mechanism for loss of gastric mucosa is understood with regard to certain precancerous conditions, such as pernicious anaemia (antiparietal-cell antibodies) and surgical interventions involving resection of segments of the organ. The most common form of atrophy, namely multifocal atrophic gastritis, however, is not understood at present.

Diets high in starch and salt induce atrophy in mice, and the trophic effects of hormonal imbalance have been offered as an explanation (Kodama *et al.*, 1984). Deficient repair of cell loss after gastritis, due to inadequate protein nutrition, has also been considered.

#### *Genotoxic agents*

The key event in the proposed chain of causation is the delivery of a genotoxic agent in small doses over a prolonged period of time to the gastric mucosa. It has been postulated that such genotoxic agents are *N*-nitroso compounds synthesized *in situ*. So far, this hypothesis is based on circumstantial evidence: (i) high nitrite levels in the gastric juice of high-risk populations; (ii) experimental carcinogenesis after administration of nitrite and amines in the diet; (iii) positive correlations between nitrate intake and cancer risk in some populations; (iv) higher levels of endogenous nitrosation after feeding of proline to high-risk populations, which suggest that endogenous nitrate formation is possibly associated with gastritis (Kamiyama *et al.*, this volume). Some attempts to test this part of the hypothesis have given negative results. One of them reports a negative correlation between nitrate intake and cancer risk in England (Forman *et al.*, 1985). The variable results of correlation studies are illustrated in Table 2, in which pH, nitrate and nitrite levels are compared by histopathology of gastric biopsies in two populations: peasants of Nariño, Colombia, with a very high gastric cancer incidence rate ( $150 \times 10^5$ ), and blacks of New Orleans, USA, with a rate double that of whites of the same city ( $20 \times 10^5$ ), but much lower than the Nariño rate. For all categories of gastric pathology, nitrate levels are much higher in Colombia, and a correlation analysis between these two populations will be positive. Within Colombia, nitrate levels increase with the severity of gastric lesions — again, a positive correlation with risk. Within New Orleans, however, more advanced lesions are associated with lower levels of nitrate — a negative correlation similar to that reported from England. The most logical explanation for these findings is that dietary nitrate in Colombia occurs mostly in foods associated with increased cancer risk, such as starchy foods (mostly potatoes) and grains, such as fava beans. By contrast, in New Orleans, dietary nitrate occurs mostly in fresh fruits and vegetables, the role of which in decreasing cancer risk is amply documented. In high-risk populations, nitrite results mostly from reduction of gastric nitrate, and it has been shown that increased intake of nitrate by patients with gastritis results in a dramatic increase in nitrite in the gastric juice (Eisenbrand *et al.*, 1984a). In high-risk populations, therefore, an increase in nitrate supply definitely increases the chances of nitrosation reactions, by increasing nitrite availability. Nitrate supply is not the limiting factor in populations at low or intermediate risks, however, since practically all human diets contain enough nitrate, which is available for reduction under the right circumstances (CAG). In the absence of CAG, the concentration of nitrate in the stomach does not determine the amount of nitrite available.

In-vivo nitrosation, as evaluated by the Ohshima-Bartsch test, is not excessive in CAG, primarily because an acid gastric pH (not present in CAG) is a prerequisite of proline nitrosation. Other models of nitrosation based on higher pH levels will be more pertinent (Keefer & Roller, 1973) but are not available for human testing. *N*-Nitrosoproline can be ruled out as a human gastric carcinogen, but the search for a specific human gastric *N*-nitroso carcinogen is bewildering, given the enormous number of compounds and the difficulties of working with directly-acting *N*-nitroso carcinogens. Two very relevant candidates, however, are available which illustrate the optimal relationship between epidemiology and laboratory sciences. Weisburger and coworkers, using Japanese epidemiological data on food consumption, nitrosated a fish commonly used in the Japanese diet and with it induced adenocarcinoma of the glandular stomach in experimental

**Table 2. Mean<sup>a</sup> levels of gastric juice pH, nitrate and nitrite in two populations: New Orleans, Louisiana, and Nariño, Colombia**

Component	Normal	Superficial gastritis	Intestinal metaplasia	Dysplasia
<b>pH</b>				
Louisiana	3.1 (2.13, 4.13)	3.5 (2.83, 4.25)	4.3** (3.63, 4.83)	5.0** (3.92, 6.0)
Colombia	3.2 (2.94, 3.42)	4.0 (3.64, 4.18)	6.0** (5.59, 6.26)	7.0** (6.55, 7.55)
<b>Nitrate (<math>\mu</math>M)</b>				
Louisiana	254.79 (214.88, 302.12)	185.10* (120.39, 284.61)	146.95** (95.17, 226.91)	118.25** (58.41, 239.39)
Colombia	368.19 (310.51, 436.58)	475.79* (403.73, 560.70)	554.03** (435.73, 704.45)	855.96** (609.35, 1202.40)
<b>Nitrite (<math>\mu</math>M)</b>				
<b>pH &lt; 5.0</b>				
Louisiana	1.16** (1.10, 1.21)	1.79** (1.51, 2.12)	1.29** (1.16, 1.44)	1.16 (0.84, 1.59)
Colombia	1.0** (0.99, 1.01)	1.05** (0.98, 1.12)	1.0** (0.93, 1.08)	1.05 (0.77, 1.44)
<b>pH 5.0+</b>				
Louisiana	-	4.72 (0.91, 24.33)	4.08** (1.70, 9.80)	4.96 (1.07, 23.06)
Colombia	6.92 (3.51, 13.64)	8.71 (5.33, 14.24)	14.91** (10.29, 21.62)	18.06 (9.77, 33.38)

<sup>a</sup>Adjusted for age and sex\*, Significant difference between regions,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; ( ), 95% confidence limits

rats (Weisburger *et al.*, 1980). The carcinogen responsible for these results should be identified because it may be relevant to human gastric cancer etiology. Tannenbaum and coworkers (Yang, D. *et al.*, 1984), using epidemiological data from Colombia that showed a high intake of fava beans by high-risk populations, identified a nitroschloroindol mutagen after nitrosating fava beans. This compound is very potent as a mutagen, but its carcinogenic potential has not been evaluated. It should be very relevant in the search for in-situ synthesis of *N*-nitroso carcinogens in humans.



### *Antioxidants*

Promotion is an important part of carcinogenesis, but no well-defined 'promoter' has been identified in human gastric carcinogenesis. What has been consistent in epidemiological findings is the protective role of fresh fruits and vegetables. Attempts to identify specific protective micronutrients have disclosed interpopulation differences. A strong protective effect of vitamin C is suggested in some populations (Correa *et al.*, 1985; Fontham *et al.*, 1986), while an effect of carotenoids and tocopherols is suggested in others (Haenszel *et al.*, 1985). This again indicates interpopulation differences in specific aspects of the causation chain. Vitamin C blocks nitrosation — a key point in the hypothesis. It also promotes fibroplasia, which may play a role in the progression and spread of early cancers. A similar antioxidant role for tocopherol in lipid media is well known. The mode of action of carotenoids is less clear: they may act as retinol precursors, but the lack of a protective role of retinol does not support such a mechanism. Two recent pieces of evidence suggest that carotenoids (and not retinol) may act as scavengers of free radicals. Experimental administration of  $\beta$ -carotene to rats previously treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine had no effect on the appearance of precancerous lesions but inhibited the late stages of the carcinogenic process (Santamaria *et al.*, 1986). Similarly, low blood levels of carotenoids are seen in human subjects with advanced precancerous lesions (dysplasia) but not in subjects with normal mucosa or earlier precancerous lesions such as CAG and intestinal metaplasia (Haenszel *et al.*, 1985).

### *Genetics*

Although there is abundant evidence for the role of environmental factors in CAG causation, genetic mechanisms are involved in determining susceptibility to the disease. Recent segregation analysis of Colombian families indicates the influence of a major CAG susceptibility recessive gene which is transmitted autosomally. The expression of this gene increases with age and is also influenced by having an affected mother, indicating the interaction between genetics and the environment (Bonney *et al.*, 1986).

### **Epilogue**

The etiological model of gastric cancer illustrates well the complexities of human carcinogenesis. While it is highly predictable that administration of 250  $\mu\text{g/ml}$  *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in drinking-water will induce gastric adenocarcinoma in inbred rats fed a uniform diet, it should be obvious that such high doses and this degree of environmental and genetic homogeneity are far removed from the human situation. The gastric cancer model may be more representative of other human cancers. The overriding effect of cigarette smoking in lung cancer causation may not represent the situation for other cancers because the dose of the carcinogen is considerable. Recently, however, even in this straightforward example of human carcinogenesis, the modulating role of nutrition has been recognized (Byers *et al.*, 1984; Hinds & Kolonel, 1984; Ziegler *et al.*, 1986). In-situ nitrosation of tobacco-specific nitrosamines by salivary nitrite may be the best available evidence for the role of carcinogenic *N*-nitroso compounds in humans (Brunnemann *et al.*, this volume) and again points to the relevance of human models based on in-situ nitrosation.

The greatest difficulty in epidemiological exploration of the role of *N*-nitroso compounds in human carcinogenesis is the inability to document exposure. It is indeed remarkable that by using tools as weak as dietary questionnaires epidemiologists have been

able to provide such convincing evidence of the protective role of substances like carotenoids. This proves that the epidemiological techniques are available and ready to be applied with better markers of exposure. This is the case for tumours in which markers (so far, mostly antibodies) provide evidence of past exposure to biological agents, such as hepatitis B virus and HTLV-I. In comparison to these viruses, *N*-nitroso compounds have much simpler structures and cannot be expected to induce antibodies by themselves; but other markers of exposure may be available, such as the DNA adducts discussed at this meeting.

Detecting markers of exposure to *N*-nitroso compounds may be the single greatest service that experimentalists can provide to epidemiologists in the collaborative effort to establish the role of these compounds as a cause of human cancer.

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## CONSUMPTION OF PRECURSORS OF N-NITROSO COMPOUNDS AND HUMAN GASTRIC CANCER

N.W. Choi<sup>1</sup>, A.B. Miller<sup>2</sup>, J.G. Fodor<sup>3</sup>, M. Jain<sup>2</sup>, G.R. Howe<sup>4</sup>,  
H.A. Risch<sup>2</sup>, & A.M. Ruder<sup>1</sup>

<sup>1</sup>Department of Epidemiology and Biostatistics, Manitoba Cancer Treatment and Research Foundation, and University of Manitoba, Winnipeg, Manitoba; <sup>2</sup>NCIC Epidemiology Unit, Department of Preventive Medicine and Biostatistics, University of Toronto, Toronto, Ontario; and <sup>3</sup>Faculty of Medicine, Memorial University of Newfoundland, St John's, Newfoundland, Canada

It has been hypothesized that dietary nitrate and nitrite are converted in the stomach to nitrous acid, which reacts with secondary amines and amides to form nitrosamines and nitrosamides, compounds frequently demonstrated to be carcinogenic in animals, and that vitamins C and E inhibit *N*-nitroso product formation by chemically reducing nitrous acid. This hypothesis and others were tested in a case-control study (controls were individually matched by age, sex and area of residence), utilizing a standardized, quantitative, dietary history questionnaire interview. Daily nutrient consumption values were calculated from interview responses, and continuous conditional logistic regression was used for the data analysis. Significant findings are as follows: (1) Average daily consumption of nitrite, chocolate and carbohydrate was associated with increasing trends in risk. (2) While citrus fruit intake appeared to be somewhat protective, any protective effect of vitamin C intake was less apparent, and of vitamin E, not at all apparent. (3) Consumption of dietary fibre was negatively associated with gastric cancer risk. These findings appear to implicate a number of dietary components, including nitrite consumption, in the genesis of gastric cancer in humans.

The incidence of and mortality from gastric cancer vary greatly from country to country, between migrating populations and their former compatriots, and between generations within some migrant racial groups. Numerous past studies of gastric cancer in migrant populations point to the importance of environmental factors, and particularly of dietary factors, in the genesis of the cancer. Among many such studies, those carried out in Manitoba, Canada, on the ethnic distribution of cancer of the gastrointestinal tract (Table 1; Choi, 1968) and on changes in dietary habits between generations of Icelandic migrants in Manitoba (Choi *et al.*, 1971) suggest the importance of certain ethnic foods, such as smoked, pickled and cured meats and fish, in the genesis of gastric cancer (Table 2). These foods are known to contain carcinogenic agents such as aromatic hydrocarbons and *N*-nitroso compounds.

It has been hypothesized that dietary nitrate and nitrite are converted in the stomach to nitrous acid, which then reacts with secondary amines and amides to form nitrosamines and nitrosamides. These compounds have frequently been demonstrated to be carcinogenic in animals (Sander *et al.*, 1975; Mirvish, 1983). Water, especially well-water, may also contain nitrates (National Research Council, 1981). In unrefrigerated food, bacteria can convert

**Table 1. Standardized mortality ratios with numbers of deaths from stomach cancer according to ethnic origin by foreign-born and native-born by sex, Manitoba residents, 1956-1965<sup>a</sup>**

Ethnic origin		Foreign born		Native born	
		Observed deaths	SMR <sup>b</sup>	Observed deaths	SMR <sup>b</sup>
Scandinavian	Male	53	248 (185-326)	29	178 (119-256)
	Female	21	282 (175-431)	11	135 (67-242)
Icelandic	Male	23	391 (248-587)	16	186 (106-301)
	Female	10	316 (152-581)	7	166 (66-342)

<sup>a</sup> From Choi (1968)<sup>b</sup> In parentheses, 95% confidence limits**Table 2. Groups of foods eaten more frequently by Iceland-born residents of Manitoba, aged 65 and over (based on a score computed for each individual<sup>a</sup>) ( $\alpha = 0.05$ )<sup>b</sup>**

Group of foods	Icelandic (score)						Non-Icelandic (score)					
	1	2	> 2	1	2	> 2	1	2	> 2	1	2	> 2
<i>Skyr</i>	30	27.3	29	26.4	51	46.4	101	53.4	50	26.5	38	28.1
Salted and pickled meats	30	27.3	49	44.5	31	28.2	96	50.8	58	30.7	35	18.5
Soured meats	53	48.2	24	21.8	33	30.0	152	80.4	22	11.6	15	7.9
Smoked and singed foods	27	24.5	36	32.7	47	42.7	87	46.0	63	33.3	39	20.6

<sup>a</sup> Each food group score for an individual was obtained by adding together the individual's frequency of eating categories, before and after the Second World War, of all foods in the group and dividing by an integer (Categories are numbered 0, 1, 2, 3, for never, seldom, often and very often, respectively.)<sup>b</sup> From Choi *et al.* (1971)

nitrites (Weisburger & Raineri, 1975). Preformed nitrosamines have been found in some foods, but most are formed in the stomach, depending on stomach pH and other factors (National Academy of Sciences, 1981).

The human diet appears to include inhibitors as well as promoters of carcinogenesis: vitamins C and E have been shown to inhibit *N*-nitroso product formation by chemically reducing nitrous acid (Mirvish, 1983). A case-control study of diet and gastric cancer was designed to test these hypotheses of cancer promotion and inhibition and to determine the relative risk of ingesting particular food constituents.

## Methods

A multicentre collaborative case-control study was conducted during 1979-1982 in Toronto (Ontario), Winnipeg (Manitoba) and St John's (Newfoundland), Canada. Individuals between 35 and 79 years old, newly diagnosed with gastric cancer, were identified in Manitoba and Newfoundland through the provincial tumour registries and in Toronto, through periodic examination of surgical, pathology and medical records in those area hospitals where stomach cancer cases are treated. Of the 565 eligible patients, 250 (44%) were interviewed after initial contact through their physicians. Four cases were excluded owing to unreliable interviews. Controls, identified through door-to-door searches, electoral lists, street directories and municipal enumeration lists, were individually matched to cases for age, sex and area of residence. Of the eligible controls, 58% (250 of 429) agreed to be interviewed; four were excluded, corresponding to the excluded cases. All interviews utilized a standardized, quantitative dietary history questionnaire (Morgan *et al.*, 1978; Jain *et al.*, 1980) and a personal and medical history questionnaire. For the cases, a histology form was completed from hospital records. Then, daily nutrient consumption values were calculated through use of the US Department of Agriculture (1972) Food Composition Data Bank, which was extended and modified for Canadian items (Arthur, 1972; Panalaks *et al.*, 1973, 1974; Gray *et al.*, 1979; McLaughlin & Weihrauch, 1979; National Academy of Sciences, 1981). Continuous conditional logistic regression methods for matched studies (Breslow & Day, 1980) were used for the data analysis to account for the simultaneous and possibly confounding multiple exposures.

## Results and discussion

Risk increased with increasing average daily consumption of nitrite-rich foods and decreased with increasing consumption of nitrates, vitamin C and citrus fruits (Table 3), while *N*-nitrosodimethylamine and vitamin E did not appear to affect risk. An analysis of simultaneous consumption of nitrate and ascorbate in 21 common vegetables (Table 4) shows an increased risk for nitrate, instead of the protective effect seen in Table 3, and a more significant reduction in risk for vitamin C. In Tables 3 and 4, each dietary factor was analysed separately in a model which also included total food consumption and ethnicity. In a multivariate analysis simultaneously incorporating high-fibre and high-nitrite foods, as well as total food consumption and ethnicity, nitrites, chocolate and carbohydrate were associated with increasing trends in risk (Table 5). When ascorbate and citrus fruits, which appeared to be somewhat protective when analysed separately, were added to the model of Table 5, they had no significant effect on risk. In the multivariate model, only the consumption of dietary fibre was negatively associated with gastric cancer risk (Table 5).

These findings appear to implicate a number of dietary components, including nitrite consumption, in the genesis of gastric cancer in humans. However, some limitations must be noted. Only 44% of eligible cases participated in the study. Many cases may have been excluded because of early death or severe disease; our findings may represent individuals who were diagnosed at earlier stages or with less severe forms of disease. In addition, gastric cancer patients, as opposed to controls, may have overreported dietary information. It was for this reason that total food consumption was included in all the regression models. We were not able to group case responses by histological type or physical location. Ethnicity was included in the models to represent genetic or familial exposures, because of the known

**Table 3. Trends in gastric cancer risk with nitrite-related factors<sup>a</sup>**

Factor	Odds ratio	Unit	95% Confidence interval	p value
Nitrite	1.71	1 mg/day	1.24-2.37	0.00061
Smoked meats	2.22	100 g/day	1.19-4.15	0.0077
Smoked fish	2.03	100 g/day	0.34-12.2	NS
Nitrate	0.66	100 g/day	0.54-0.81	0.00002
N-Nitrosodimethyl-amine	0.94	10 g/day	0.14-6.13	NS
Vitamin C	0.43	1 g/day	0.15-1.20	0.099
Citrus fruit	0.75	100 g/day	0.60-0.92	0.0056
Vitamin E	1.01	100 mg/day	0.89-1.14	NS
No refrigeration	1.19	10 years	1.04-1.35	0.0072
Public water supply	0.86	10 years	0.76-0.99	0.029

<sup>a</sup>Each model also includes total food consumption and ethnicity (British Isles, Oriental, other). In a preliminary analysis, only English ethnicity significantly reduced risk — odds ratio, 0.50 (0.31-0.80). Oriental (Chinese, Japanese, Korean) ethnicity increased risk — odds ratio, 3.09 (0.97-9.86) — but was not significant in a multiple comparison.

NS, not significant

**Table 4. Trends in gastric cancer risk with ascorbate and nitrate<sup>a</sup>**

Factor	Odds ratio	Unit (mg/day)	95% Confidence interval	$\chi^2$	df <sup>b</sup>	p value
Ascorbate	0.149	100	(0.033-0.58)	8.05	2	0.018
Nitrate	1.63	100				
All 21 vegetables	(lack of fit of above model)			15.72	19	NS

<sup>a</sup>Each model also includes total food consumption and ethnicity. The 21 vegetables are lettuce, spinach, endive, radish, Brussels sprout, rutabaga, sweet pepper, artichoke, asparagus, okra, beetroot greens, chard, cucumber, zucchini, cauliflower, broccoli, cabbage, kale, pea, turnip and turnip greens.

<sup>b</sup>df, degrees of freedom

NS, not significant

association of ABO blood types and gastric cancer (Bjelke, 1980). If differing gastric cancer risks for various ethnic groups were due to different typical diets, the ethnic factor would bias the analysis of dietary component risks. However, since ethnicity odds ratio estimates did not change greatly when food components were added to the models, ethnicity appears to represent nondietary factors.

Our results are consistent with those of previous studies of diet and gastric cancer. Increased risk with consumption of smoked meats and fish has been shown in a number of studies (Meinsma, 1964; Higginson, 1966; Haenszel *et al.*, 1972, 1976; Bjelke, 1979; Juha'sz, 1980). Our finding of increased risk with chocolate consumption is supported

**Table 5. Trends in gastric cancer risk with calculated consumption of food constituents<sup>a</sup>**

Factor	Odds ratio	Unit	Confidence interval	<i>p</i> value
Dietary fibre <sup>b</sup>	0.40	10 g/day	0.28-0.58	<10 <sup>-8</sup>
Nitrite	2.61	1 mg/day	1.61-4.22	<10 <sup>-4</sup>
Chocolate	1.84	10 g/day	1.22-2.77	<10 <sup>-4</sup>
Carbohydrates	1.53	100 g/day	1.07-2.18	0.015
No refrigeration	1.17	10 years	1.01-1.35	0.037

<sup>a</sup>Model simultaneously includes all factors shown, as well as total food consumption and ethnicity

<sup>b</sup>Dietary residual after digestion by stomach enzymes, etc  
NS, not significant

by the work of Modan *et al.* (1974) and by those of Haenszel *et al.* (1972) and Jedrychowski *et al.* (1980), who observed increased risk with consumption of sweets. The protective effect of citrus fruit seen in our study has been observed by some researchers (Meinsma, 1964; Higginson, 1966; Bjelke, 1979) but not by others (Acheson & Doll, 1964; Graham *et al.*, 1972; Haenszel *et al.*, 1972).

Our results, in agreement with those of previous studies, strongly support the hypotheses that nitrite intake is associated with increased stomach cancer risk and that consumption of citrus fruits appears to be somewhat protective. The strong trends for increased risk with chocolate consumption and decreased risk for fibre consumption suggest that much remains to be elucidated about the complex role of dietary components in carcinogenesis.

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# URINARY EXCRETION OF *N*-NITROSAMINO ACIDS AND NITRATE BY INHABITANTS IN HIGH- AND LOW-RISK AREAS FOR STOMACH CANCER IN NORTHERN JAPAN

S. Kamiyama<sup>1</sup>, H. Ohshima<sup>2</sup>, A. Shimada<sup>1</sup>, N. Saito<sup>1</sup>,  
M.-C. Bourgade<sup>2</sup>, P. Ziegler<sup>2</sup> & H. Bartsch<sup>1</sup>

<sup>1</sup>*Department of Hygiene, Akita University School of Medicine,  
Akita, Japan; and* <sup>2</sup>*International Agency for Research on Cancer,  
150, cours Albert Thomas, Lyon, France*

Three samples of 24-h urine were collected from each of 104 inhabitants of high-risk (Akita) and low-risk (Iwate) areas for stomach cancer in northern Japan, according to the following protocols: (i) when they were undosed, (ii) after ingestion of proline three times a day and (iii) after ingestion of proline together with vitamin C three times a day. These samples were analysed for *N*-nitrosamino acids, nitrate and chloride ion as indices of the exposure. The median values of *N*-nitrosoproline (NPRO) and *N*-nitroso-2-methylthiazolidine 4-carboxylic acid (NMTCA) excreted in the urine of undosed subjects were not different between the two areas; however, that of *N*-nitrosothiazolidine 4-carboxylic acid (NTCA) was significantly higher in subjects of the high-risk area. Salt intake, estimated from the level of chloride ion in the urine, did not differ in the two areas. After intake of proline, the NPRO level increased significantly only in subjects of the high-risk area, but not in those of the low-risk area; intake of vitamin C inhibited this increase of NPRO and lowered the levels of other nitrosamino acids only in the high-risk subjects. In contrast, the urinary level of nitrate was higher in subjects of the low-risk area than in those of the high-risk area; nitrate levels were found to correlate well with the amounts of vegetables consumed. These results indicate that, although nitrate intake by subjects in the high-risk area is lower, their potential for endogenous nitrosation (possibly intragastric nitrosation) is higher, suggesting the possible occurrence of some inhibitory factors for nitrosation in the diet of the low-risk area. The role of nitrate exposure and endogenous nitrosation as possible etiological factor for human stomach cancer is discussed.

Stomach cancer is among the major causes of death from cancer in Japan and various other countries in Asia, South America and eastern Europe, although its incidence is decreasing in most areas. Intragastric formation of *N*-nitroso compounds, particularly *N*-nitrosamides, has been postulated as one of the possible etiological factors for human stomach cancer (Correa *et al.*, 1975; Mirvish, 1983). However, no convincing epidemiological evidence has so far been presented, mainly due to lack of reliable data for assessing endogenous nitrosation in humans. In the present study, exposure to *N*-nitroso compounds, ingested in foods or formed endogenously, was compared in inhabitants in high- and low-risk areas for stomach cancer in northern Japan, by determining urinary levels of *N*-nitrosamino acids as the exposure indices (Bartsch *et al.*, 1983a; Ohshima *et al.*, 1985).



### Study subjects and collection of urine samples

Samples of 24-h urine were collected in the spring of 1983 from 104 healthy subjects living in Akita (a high-risk area for stomach cancer) and Iwate (a low-risk area). The age-adjusted mortalities from stomach cancer per 100 000 population for the period of 1969-1978 were 101 for males and 52 for females in Akita and 33 for males and 14 for females in Iwate. Characteristics of the study subjects are shown in Table 1. Three specimens of 24-h urine were collected from each subject, as described in the legend to Table 3. Analyses of *N*-nitrosamino acids and nitrate were performed as reported previously (Lu *et al.*, 1986). Chloride ion in the urine was determined as an index for salt intake. Study subjects were asked to complete a questionnaire to obtain information on demography, food items and beverages consumed and number of cigarettes smoked during the 24-h of urine collection (Table 2). Statistical analysis was carried out by the Wilcoxon test using a computer with the statistical package BMDP (Dixon, 1983).

**Table 1. Characteristics of study populations**

Study area	No. of subjects	Median age (years; range)	Blood group				Smoking habit		Socioeconomic class			Family history of stomach cancer		Water source	
			A	B	AB	O	Smoker	Non-smoker	High	Middle	Low	Yes	No	Aque-duct	Well-duct
<b>High-risk area (Akita)</b>															
Male	26	49 (29-66)	13	2	2	7	17	9	0	8	13	9	17	26	0
Female	26	47 (24-60)	9	8	3	5	0	26	0	10	6	7	18	26	0
<b>Low-risk area (Iwate)</b>															
Male	25	51 (21-60)	4	8	3	7	16	9	1	13	10	1	23	11	14
Female	27	49 (28-60)	7	12	3	5	1	26	0	15	6	2	24	14	13

### Urinary levels of *N*-nitrosamino acids

Median (with 95% confidence intervals) volumes of the 24-h urine samples and the amounts of *N*-nitrosamino acids ( $\mu\text{g}/\text{day}$ ), nitrate ( $\text{mg}/\text{day}$ ) and sodium chloride ( $\text{g}/\text{day}$ ) that were detected in the 24-h urine samples of subjects in the six groups are summarized in Table 3.

The levels of NPRO and NMTCA detected in the urine of the undosed subjects (groups AA and IA) were not significantly different between the two areas; the NTCA level was higher in the high-risk subjects than in the low-risk subjects ( $p < 0.05$ ). The urinary levels of NPRO and NTCA detected in the undosed specimens in northern Japan were compatible with those detected in the urine of subjects living in a high-risk area for oesophageal cancer in northern China (Lu *et al.*, 1986), however, those of NMTCA and nitrate were higher in the Japanese urines.

**Table 2. Food items and beverages consumed on the day of urine collection**

Study area	No. of subjects	No. of subjects who consumed food items									
		Meat	Fish	Ham or sausage	Egg	Chinese noodles	Tofu (soya bean cake)	Dried squid product	Boiled vegetables	Fried vegetables	Pickled or salad vegetables
<b>High-risk area (Akita)</b>											
Group AA (undosed)	52	32	43	8	24	5	6	1	43	9	32
Group AB (proline)	52	28	47	8	23	5	11	0	42	9	36
Group AC (proline + vitamin C)	52	33	41	7	14	4	7	0	39	8	34
<b>Low-risk area (Iwate)</b>											
Group IA (undosed)	52	20	44	6	36	3	19	0	48	8	34
Group IB (proline)	52	23	44	10	26	7	20	1	47	4	36
Group IC (proline + vitamin C)	52	22	48	5	24	6	20	3	48	8	38

Study area	No. of subjects who consumed food items	No. of subjects who consumed food items																	
		Pickled Radish	Turnip	Cucumber	Chinese cabbage	Egg-plant	Nozawana	Mix-ture	Others	Fruit	Milk	Coffee	Green tea	Fruit juice	Sake	Distilled sake	Beer	Whisky	Wine
<b>High-risk area (Akita)</b>																			
Group AA	5	7	20	9	9	4	0	1	3	33	24	8	16	6	8	1	8	3	1
Group AB	9	12	12	5	9	0	2	6	24	23	12	15	5	10	0	7	1	1	1
Group AC	5	11	20	5	10	0	2	9	16	24	10	15	8	5	1	9	1	0	0
<b>Low-risk area (Iwate)</b>																			
Group IA	18	10	21	15	0	5	3	1	25	13	10	12	13	8	2	6	2	1	1
Group IB	14	15	17	9	1	3	5	0	23	19	8	14	15	7	0	8	3	1	1
Group IC	10	14	25	18	1	1	3	0	23	21	12	11	17	10	1	4	2	0	0

**Table 3. Median (and 95% confidence intervals) for volumes of 24-h urine and amounts of *N*-nitrosamino acids, nitrate and sodium chloride detected in urine and *P* values<sup>a</sup> of comparison**

Study area	No. of subjects	Volume of 24-h urine (l; range)	<i>N</i> -Nitrosamino acid ( $\mu\text{g}$ /person per day)				Nitrate <sup>b</sup> (mg/person per day)	Sodium chloride (g/person per day)
			NPRO	NTCA	NMTCA	Sum		
<b>High-risk area (Akita)</b>								
Group AA (undosed)	52	1.55(0.54-3.23)	3.8(2.6-5.0)	12.4(7.7-17.1)	3.2(2.1-4.3)	20.2(14.7-26.6)	95(73-117)	14.5(12.8-16.2)
Group AB (proline)	52	1.77(0.90-3.64)	12.6(8.5-16.7)	19.0(15.5-22.5)	4.2(1.9-6.5)	43.9(33.7-54.0)	116(83-150)	15.4(13.4-17.5)
Group AC (proline + vitamin C)	52	1.83(0.94-3.18)	3.2(2.4-4.0)	7.2(4.9-9.4)	2.0(0.9-3.0)	15.2(11.3-19.1)	130(107-160)	15.0(13.2-16.9)
<b>Low-risk area (Iwate)</b>								
Group IA (undosed)	52	1.39(0.60-4.11)	6.1(4.1-8.1)	5.7(2.0-9.4)	2.8(1.2-4.4)	14.8(7.4-22.2)	145(118-170)	13.6(11.8-15.4)
Group IB (proline)	52	1.34(0.52-2.90)	7.1(6.6-9.6)	11.9(8.2-15.6)	4.2(3.0-5.3)	24.3(19.4-29.2)	177(136-219)	14.0(11.8-16.2)
Group IC (proline + vitamin C)	52	1.54(0.49-2.67)	4.9(3.3-6.5)	4.9(1.1-8.9)	2.2(1.0-3.3)	15.8(9.1-22.4)	88(61-115)	14.1(12.5-15.7)

	NPRO		NTCA		NMTCA		Sum		Nitrate		NaCl	
AA vs IA	NS	0.05	NS	NS	NS	NS	NS	0.001	NS	NS	NS	NS
AB vs IB	0.001	0.005	NS	NS	NS	NS	NS	0.001	NS	NS	NS	NS
AC vs IC	0.05	0.05	NS	NS	NS	NS	NS	0.001	NS	NS	NS	NS
AA vs AB	0.001	0.05	NS	NS	NS	NS	NS	0.001	0.001	NS	NS	NS
AA vs AC	NS	0.01	NS	0.001	0.01	0.001	NS	0.005	NS	NS	NS	NS
AB vs AC	0.001	0.001	NS	0.001	0.001	0.001	NS	NS	NS	NS	NS	NS
IA vs IB	NS	0.01	NS	NS	NS	NS	NS	0.05	NS	NS	NS	NS
IA vs IC	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
IB vs IC	NS	NS	NS	NS	NS	NS	NS	NS	0.001	NS	NS	NS

<sup>a</sup> *P* values of comparison (NS, not significant)

<sup>b</sup> Medians (95% confidence intervals) for all Akita subjects and for all Iwate subjects are 116 (104-128) and 140 (117-163) mg/person/day, respectively; differences, *P* < 0.07

Three urine specimens were collected from each subject according to the following protocols: (i) undosed specimens of 24-h urines (Group AA and IA) were collected in order to determine the background levels of *N*-nitrosamino acids, nitrate and chloride ion; (ii) proline specimens (Group AB and IB) were collected the day the subjects had ingested 100 mg L-proline three times a day 1 h after each meal; (iii) proline plus vitamin C specimens (Group AC and IC) were collected the day they had ingested 100 mg L-proline together with 100 mg ascorbic acid three times a day 1 h after each meal

Intake of proline (100 mg three times a day) resulted in a significant increase in the urinary NPRO excretion in the subjects of the high-risk area (from 3.8 to 12.8  $\mu\text{g}$ /person per day,  $p < 0.001$ ), but not in those of the low-risk area (from 6.1 to 7.1  $\mu\text{g}$ /person per day), compared with the NPRO level in the undosed specimens. These results indicate that potential for endogenous nitrosation is higher in the subjects of the high-risk area. Intake of moderate doses of ascorbic acid together with proline markedly decreased the urinary levels of NPRO and other *N*-nitrosamino acids in the subjects of the high-risk area (group AC), compared to the levels of the undosed (group AA) or proline specimens (group AB). However, no significant effect of ascorbic acid was observed in the subjects of the low-risk area.

#### Urinary levels of nitrate and chloride ion

The median value of nitrate for all urine samples collected in the high-risk area was 116 mg/person per day, which was significantly lower than 140 mg/person per day for all samples from the low-risk area ( $p < 0.07$ ). The level of sodium chloride was not different between the two areas or among the groups within one area.

#### Effect of smoking on urinary *N*-nitrosamino acids and nitrate

In accordance with the results of other studies (Ohshima *et al.*, 1984a; Lu *et al.*, 1986; Tsuda *et al.*, 1986), smokers excreted greater amounts of NTCA than did nonsmokers ( $p < 0.005$ ), but the levels of NPRO, NMTCA and nitrate in the undosed urine were not significantly different between smokers and nonsmokers. There was a marked tendency for greater excretion of NPRO and NMTCA with number of cigarettes smoked ( $p < 0.002$  and  $p < 0.05$ , respectively). In addition, the level of NPRO in the urine after intake of proline was significantly correlated with the number of cigarettes smoked on the day of urine collection ( $p < 0.001$ ). A similar enhancing effect of cigarette smoking on endogenous nitrosation of proline has been reported by Hoffmann and Brunnemann (1983) and Ladd *et al.* (1984b).

#### Effect of food items and beverages consumed on urinary *N*-nitrosamino acids and nitrate

Food items and beverages consumed by subjects during the day of urine collection are summarized in Table 2. It has been reported that ham and sausages contain protein-bound NPRO and that the consumption of these food results in a marked increase of urinary NPRO (Stich *et al.*, 1984a). However, in the present study the amounts of three *N*-nitrosamino acids (NPRO, NTCA, NMTCA) excreted by the subjects who had consumed ham and/or sausages did not differ from those excreted by the subjects who had not eaten these foods. No apparent relationship was observed between levels of *N*-nitrosamino acids and consumption of pickled vegetables. The urinary nitrate level was, however, highly positively correlated with the amounts of vegetable consumed, in particular those of pickled vegetables ( $p < 0.002$ ). More detailed analyses of the effects of food items on the urinary levels of *N*-nitrosamino acids and nitrate are under way.

#### Nitrate exposure, endogenous nitrosation and stomach cancer

Nitrate exposure has been positively correlated with the incidence of stomach cancer in 12 countries (Hartman, 1983) and in specific areas of several other countries, including the UK, Colombia, Chile, Denmark, Hungary and Italy (Hill *et al.*, 1973; Cuello *et al.*, 1976; Zaldivar, 1977; Juha'sz *et al.*, 1980; Amadori *et al.*, 1980). However, studies that showed no correlation or even an inverse correlation between nitrate exposure and the incidence of

stomach cancer have also been reported (Davies, 1980; Armijo *et al.*, 1981; Forman *et al.*, 1985). Armijo *et al.* (1981) reported higher levels of nitrate in the urine of school children from a low-risk area in Chile. Forman *et al.* (1985) recently showed lower levels of salivary nitrite and nitrate in healthy subjects from areas of the UK with high gastric cancer incidences than in areas with low incidences of this cancer. Similarly, the Japanese subjects in our study living in the high-risk area excreted significantly lower levels of nitrate in the urine. The present study also revealed, however, that urinary levels of nitrate correlate well with the consumption of vegetables. As Mirvish (1985) and Tannenbaum and Correa (1985) have pointed out in commenting on the results reported by Forman *et al.* (1985), vegetables contain not only nitrate but also other constituents, such as vitamin C and phenolic compounds, which generally inhibit *N*-nitrosation (Pignatelli *et al.*, 1984). In fact, the potential for endogenous nitrosation, which was estimated from an increased level of NPRO after intake of proline, was much higher in the high-risk populations. In contrast, the low-risk subjects did not excrete elevated levels of NPRO after intake of proline, and decreased levels of nitrosamino acids were not observed after intake of ascorbic acid. These results indicate that the low-risk subjects may already ingest sufficient amounts of the agents that suppress endogenous nitrosation, although their intake of nitrate is higher.

In conclusion, the process of endogenous nitrosation in humans is highly complex and is influenced by many factors, such as pH of stomach contents, the nature of nitrosatable substances and occurrence of bacteria, catalysts and inhibitors. Therefore, determination only of nitrate and nitrite in saliva, urine or gastric juice is insufficient to assess the whole process of endogenous nitrosation in humans. In addition, previous comparisons of food habits and analyses of mutagens in foods in these two areas (Shimada, 1980; Kamiyama & Michioka, 1983) suggest that some vegetables are protective against stomach cancer. In this respect, blood samples collected from the same subjects were analysed for various vitamins, trace elements and other nutritional parameters. A multi-variable statistical analysis is being carried out to study the relationships between endogenous nitrosation and nutritional factors in the etiology of stomach cancer in northern Japan. Complete results of these studies will be reported elsewhere.

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# A CORRELATION STUDY ON URINARY EXCRETION OF N-NITROSO COMPOUNDS AND CANCER MORTALITY IN CHINA: INTERIM RESULTS

J. Chen<sup>1</sup>, H. Ohshima<sup>2</sup>, H. Yang<sup>3</sup>, J. Li<sup>4</sup>, T.C. Campbell<sup>5</sup>, R. Peto<sup>6</sup>  
& H. Bartsch<sup>2</sup>

<sup>1</sup>*Institute of Nutrition and Food Hygiene, Chinese Academy of Preventive Medicine, 29 Nan Wei Rd, Beijing, China;*

<sup>2</sup>*International Agency for Research on Cancer, Lyon, France;*

<sup>3</sup>*Institute for Food Safety Control and Inspection, Ministry of Public Health, 29 Nan Wei Road, Beijing, China;*

<sup>4</sup>*Cancer Institute, Chinese Academy of Medical Sciences, Beijing, China;*

<sup>5</sup>*Division of Nutritional Sciences, Cornell University, Ithaca, NY 14853, USA; and* <sup>6</sup>*Imperial Cancer Research Fund Cancer Studies Unit, University of Oxford, Oxford, UK*

Samples of 12-h overnight urine were collected from approximately 40 male adults in each of the 26 counties of China. Two urine specimens were collected from each subject — one after a loading dose of proline and ascorbic acid and another after a loading dose of proline only. Levels of *N*-nitrosamino acids, nitrite and nitrate were measured in urine samples and correlated with cancer mortality per 100 000 male subjects in the truncated age range 35-64 years. Preliminary results show no clear correlation between presence of stomach cancer or liver cancer and nitrosation potential [as measured by the urinary level of *N*-nitrosoproline (NPRO) after the proline load test or of nitrate]. There was a moderate, although not clearly significant, tendency for oesophageal cancer mortality rates to be associated positively with nitrosation potential and negatively with background ascorbate levels in plasma. This result was due chiefly to the inclusion of one county (Song Xian) in which there is a fairly high oesophageal mortality rate, an average nitrosation potential three times greater than that of any other county, and the lowest ascorbate index of any county. Further study of this county is planned.

Exposure to endogenously and exogenously formed *N*-nitroso compounds has been suspected of being associated with increased risks of cancers of the stomach, oesophagus, liver and urinary bladder (Bartsch & Montesano, 1984). As part of an ecological study in progress in China (Chen *et al.*, 1987), the objective of the present investigation was to determine whether exposure to *N*-nitroso compounds is a significant risk factor for cancer at selected sites by using NPRO and three other *N*-nitrosamino acids excreted in the urine as exposure indices (Ohshima *et al.*, 1985; Lu *et al.*, 1986).

## Study subjects and collection and analysis of urine

Samples of 12-h overnight urine were collected in late 1984 from 1035 healthy male subjects, between 35 to 64 years of age, living in 26 rural counties of 14 provinces. These counties were selected from the 65 counties that had previously served as the basis for a 1983 survey (Chen *et al.*, 1987) intended to examine the effects of multiple dietary and other factors in cancer etiology. A wide range of mortality rates for oesophageal, gastric and liver

cancers were represented in these 26 counties. Two communes were selected for survey in each county, and 17-21 subjects were surveyed in each commune from among the 25 randomly selected male subjects who had participated in the 1983 survey. Urine samples were collected from each subject according to the following protocols: (i) for the vitamin C specimens, subjects were given 500 mg L-proline and 200 mg ascorbic acid 1 h after the evening meal; and (ii) 24 h later, for the proline specimens, only 500 mg L-proline were given. Of the subsequent 12-h individual urine samples, 5% was taken and pooled to give one sample for each commune. All pooled samples were immediately frozen and stored at  $-20^{\circ}\text{C}$  prior to analysis at IARC. *N*-Nitrosamino acids, including NPRO, *N*-nitroso-2-methylthiazolidine 4-carboxylic acid (NMTCA), *N*-nitrosothiazolidine 4-carboxylic acid (NTCA) and *N*-nitrososarcosine (NSAR), were analysed by gas chromatography coupled with a Thermal Energy Analyzer. Urinary thioethers, nitrite, nitrate and creatinine were also measured (Ohshima *et al.*, 1987). Analysis of ascorbate in plasma and urine and the ascorbic acid loading test were carried out as described by Chen *et al.* (1987). Statistical analysis involved standard regression and correlation methods.

### Relationship between exposure to *N*-nitrosamino acids and cancer mortality

Table 1 shows the amounts of *N*-nitrosamino acids and nitrate excreted in the urine of the study population after a proline load, with and without ascorbic acid. Although these amounts varied considerably, urinary NPRO levels after intake of either proline alone or proline and ascorbic acid were well correlated ( $p < 0.001$ ) in the two communes within one county. Similarly, the levels of NMTCA, NTCA and the sum of the four *N*-nitrosamino acids after intake of proline and ascorbic acid were well correlated between the two communes.

**Table 1. Amounts of *N*-nitrosamino acids (NAA) and nitrate excreted in 12-h urine<sup>a</sup>**

NAA ( $\mu\text{g}$ )	After proline load				After proline load with ascorbic acid					
	Mean $\pm$ SD	Min	Max	Within-county correlation of two communes		Mean $\pm$ SD	Min.	Max.	Within-county correlation of two communes	
				<i>r</i>	<i>p</i>				<i>r</i>	<i>p</i>
NSAR	0.35 $\pm$ 0.22	0.06	0.95	25%	NS	0.46 $\pm$ 0.38	0.05	1.81	24%	NS
NPRO	12.37 $\pm$ 10.04	2.86	45.25	63%	< 0.001	6.35 $\pm$ 7.75	0.86	39.57	91%	< 0.001
NMTCA	3.51 $\pm$ 5.93	0.03	28.16	36%	NS	1.58 $\pm$ 2.31	0.04	9.50	63%	< 0.001
NTCA	21.56 $\pm$ 16.39	5.13	67.14	33%	NS	13.28 $\pm$ 7.72	3.30	36.94	65%	< 0.001
Sum	37.79 $\pm$ 25.79	8.68	104.78	39%	NS	21.67 $\pm$ 14.84	4.77	78.77	80%	< 0.001
Sodium nitrate (mg)	260 $\pm$ 130	90	490	54%	< 0.01	-	-	-	-	-

<sup>a</sup>Mean  $\pm$  SD; minimum and maximum values in 26 counties (each estimated as the mean of the values in two communes/county)

Intake of ascorbic acid markedly reduced urinary excretion of NPRO, NMTCA and the sum of the four *N*-nitrosamino acids, indicating that endogenous formation of these compounds was inhibited by ascorbic acid, as suggested previously (Ohshima *et al.*, 1984a). Thus, the decrease in the amount of urinary NPRO excretion that was achieved

by adding ascorbic acid to the proline load, provides an indication of the 'nitrosation potential' *in vivo* (Ohshima *et al.*, 1985; Lu *et al.*, 1986).

Table 2 summarizes the correlation coefficients of cancer mortality rates with the various indices of exposure to *N*-nitroso compounds. No clear correlation was seen of any of the principal factors considered with the four types of cancer that were chiefly suspected of being associated with exposure to *N*-nitroso compounds. However, there was a tendency for the mortality rate from oesophageal cancer to be correlated positively with the 'nitrosation potential' (i.e., the decrease in the amount of urinary NPRO after adding ascorbic acid to the proline load) and to be correlated inversely with the 'ascorbate index', as defined in the legend to Table 2. Further examination of the data showed that in one county (Song Xian) in Henan Province, a high-risk area for oesophageal cancer in northern China, the 'nitrosation potential' was three times as great as in any other county. In this county, nitrate excretion was twice the general average, the 'ascorbate index' was one-third lower than in any other county, and the oesophageal cancer mortality rate was 1.3 standard deviations above average. As such, it alone was largely responsible for the suggestive correlations with oesophageal cancer seen in Table 2. The rates for stomach and for liver cancer in Song Xian county were, however, unremarkable.

**Table 2. Correlation coefficients of cancer mortality rates with various indices of potential exposure to *N*-nitroso compounds**

Cancer site	Nitrosation potential <sup>a</sup>	Background <i>N</i> -nitrosamino acid excretion <sup>b</sup>					Urinary nitrate <sup>c</sup>	Ascorbate index <sup>d</sup>
		NSAR	NPRO	NMTCA	NTCA	Sum		
Oesophagus	30%	-14%	8%	-22%	28%	15%	21%	-42%*
Stomach	-28%	-3%	-19%	5%	-8%	-13%	-31%	-27%
Liver	-17%	-21%	-15%	25%	9%	1%	1%	-19%
Colon/rectum	-12%	-23%	20%	30%	23%	27%	27%	-5%

\* $2p=0.034$ ; all other correlations,  $2p>0.1$ . NB: These correlations were not materially altered by logarithmic transformation of the disease rates and/or of the exposure indices.

<sup>a</sup>Difference between urinary NPRO output after proline load with and without ascorbate

<sup>b</sup>Urinary levels of NSAR, NPRO, NMTCA, NTCA, or sum of four *N*-nitrosamino acids after proline load test with ascorbate

<sup>c</sup>Following proline load

<sup>d</sup>Ascorbate index =  $(PA-m)/s + (UA-M)/S$ , where PA = plasma ascorbate, with mean  $m$  and standard deviation  $s$ , and UA = urinary ascorbate overspill after ascorbate load, with mean  $M$  and standard deviation  $S$

<sup>e</sup>Cancer mortality per 100 000 persons in the truncated age-range 35-64 between 1973-1975 (i.e., a decade before the biochemical observations)

In the present study, we found no clear correlation between cancer mortality rates and indices of potential exposure to *N*-nitroso compounds. However, because the number of counties studied was small, this finding does not exclude the relevance of such factors, particularly for oesophageal cancer. Correlations are a useful but preliminary way of exploring such data and generating hypotheses. When, as here, they draw attention



to one particular county, additional investigations are indicated. Further, multi-variable statistical analysis (Liu *et al.*, unpublished data) is being applied to examine the relationships between cancer mortality rates, *N*-nitrosamino acid excretion and a number of nutritional variables collected in the 1983 survey in the same populations (Chen *et al.*, 1987).

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# INVESTIGATIONS OF THE DNA-DAMAGING ACTIVITY OF HUMAN GASTRIC JUICE

S.A. Kyrtopoulos

*National Hellenic Research Foundation, Institute of Biological Research,  
48 Vassileos Constantinou Avenue, Athens 116 35, Greece*

Human gastric juice previously treated with nitrite was examined for its ability to cause *O*<sup>6</sup>-alkylguanine-type modifications to 2'-deoxyguanosine or DNA *in vitro*. Analysis by radioimmunoassay indicated that, in five out of ten cases, incubation with 5 mM 2'-deoxyguanosine resulted in the formation of 375-1350 fmol/ml *O*<sup>6</sup>-ethyl-2'-deoxyguanosine (*O*<sup>6</sup>-etdGuo) or, in one case, 110 pmol/ml *O*<sup>6</sup>-methyl-2'-deoxyguanosine (*O*<sup>6</sup>-medGuo). When gastric juice-treated calf-thymus DNA was examined for its ability to consume (through suicide repair of *O*<sup>6</sup>-alkylguanine-type damage) *O*<sup>6</sup>-alkylguanine-DNA alkyltransferase (AAT) from rat liver, eight out of eight samples could not. However, in four out of eight cases, a reduction in the rate of removal of *O*<sup>6</sup>-[<sup>3</sup>H]methylguanine from a <sup>3</sup>H-methylated DNA substrate was observed. This finding is compatible with the presence, in gastric juice-treated DNA, of damage capable of binding to, but not undergoing repair by, the AAT.

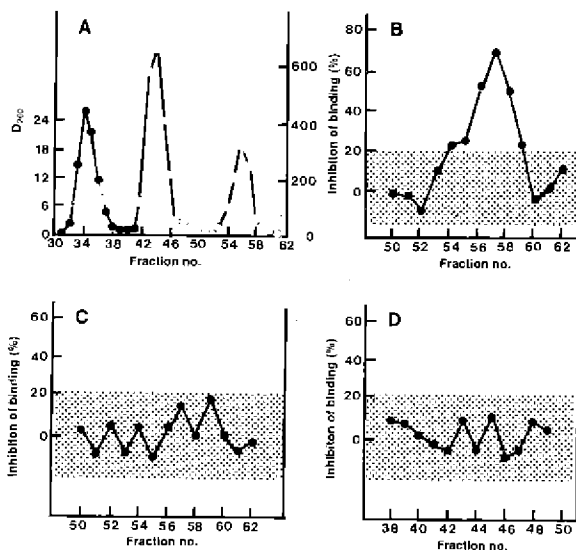
DNA alkylation plays an important role in the carcinogenesis of *N*-nitroso compounds, one of the most important precarcinogenic lesions being *O*<sup>6</sup>-alkylguanine. In view of the possible involvement of *N*-nitroso compounds in gastric juice in the etiology of human gastric cancer, and with a view to a subsequent systematic search for possible *N*-nitroso compound-induced damage in human DNA, we have examined in a pilot study the ability of nitrite-treated human gastric juice to cause *O*<sup>6</sup>-alkylguanine-type modifications of DNA, utilizing two techniques: radioimmunoassay for *O*<sup>6</sup>-medGuo and *O*<sup>6</sup>-etdGuo and competition with a <sup>3</sup>H-methylated DNA substrate for *in-vitro* repair by rat liver AAT.

## Alkylation of 2'-deoxyguanosine by nitrite-treated gastric juice

In order to maximize the probability of causing *N*-nitroso compound-related alkylation, fasting human gastric juice, previously treated under acid conditions with nitrite, was employed in the present study (see caption to Figure 1). 2'-Deoxyguanosine was used as substrate for alkylation, instead of DNA, in order to achieve a relatively high concentration of nucleophile (5 mM 2'-deoxyguanosine corresponds to over 8 mg/ml DNA). Each reaction mixture was finally analysed by combined liquid chromatography-radioimmunoassay using specific rabbit antibodies for *O*<sup>6</sup>-medGuo and *O*<sup>6</sup>-etdGuo (Kyrtopoulos & Swann, 1980). The reliability of the method employed was confirmed in separate experiments with mixtures containing exogenously added *O*<sup>6</sup>-medGuo or *O*<sup>6</sup>-etdGuo, *N*-methyl- or *N*-ethyl-*N*-nitrosourea, or methyl urea plus nitrite. Full experimental details will be published elsewhere.

Figure 1A shows a typical chromatographic profile illustrating the resolution achieved, while Figures 1B-1D show typical results obtained with two samples of gastric juice, one of which (Fig. 1B) was positive and one negative (Fig. 1C) for ethylating activity and both negative for methylating activity (Fig. 1D).

**Fig. 1. Combined liquid chromatography-radioimmunoassay analysis of gastric juice—2'-deoxyguanosine mixtures**



Gastric juice (4 ml) treated for 1 h at 0°C with 0.1 mM sodium nitrite at pH 2, was rapidly adjusted to pH 7.3 and 2'-deoxyguanosine was immediately added at 5 mM. After incubation for 24 h at 37°C in the presence of 10 μM 2'-deoxycoformycin, proteins were precipitated with 10 volumes of *n*-propanol (ethanol was avoided in order to minimize the possibility of artefactual ethylation). The mixture was dried under vacuum, redissolved in 2 ml 10 mM ammonium bicarbonate, pH 8, and 1 ml was chromatographed on a column of Aminex A7 at 50°C using the same buffer. Fractions 50-62 (1 ml each), corresponding to the *O*<sup>6</sup>-etdGuo peak, were individually vacuum-dried and analysed by radioimmunoassay. Fractions 38-49, corresponding to the *O*<sup>6</sup>-medGuo peak and the tail of the 2'-deoxyguanosine peak, were pooled, vacuum-dried, rechromatographed as above, and finally individually vacuum-dried and analysed by radioimmunoassay. A sample was considered positive for alkylated nucleoside if at least three consecutive fractions, corresponding to the anticipated peak, showed at least 20% inhibition of binding in the radioimmunoassay. A, Mixture spiked with <sup>3</sup>H-labelled *O*<sup>6</sup>-medGuo and *O*<sup>6</sup>-etdGuo; B, gastric juice no. 2, positive for *O*<sup>6</sup>-etdGuo; C, gastric juice no. 11, negative for *O*<sup>6</sup>-etdGuo; D, gastric juice no. 11, negative for *O*<sup>6</sup>-medGuo. The shaded areas delineate the limits of significance.

Ten samples of gastric juice have been examined by this method. Five were found to be positive for ethylating activity (giving rise to 1.35, 0.85, 0.27, 0.26 and 0.37 pmol/ml *O*<sup>6</sup>-etdGuo and an additional one for methylating activity (giving rise to 110 pmol/ml *O*<sup>6</sup>-medGuo). The samples analysed had been obtained from achlorhydric or normal individuals, with or without a history of gastroduodenal disease. No correlation between alkylating activity and any of the characteristic parameters of the gastric juice samples was observed.

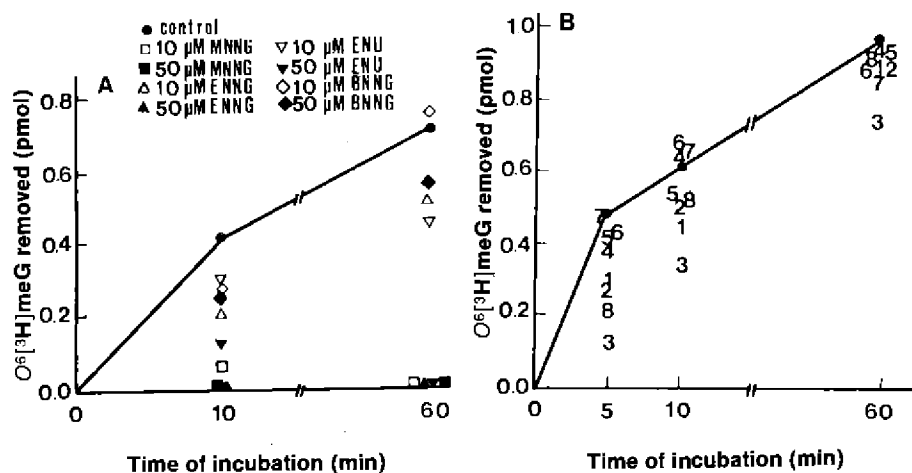
We have no indication as to the possible chemical nature of the alkylating species involved. Because, for practical reasons, the gastric juice had been obtained from individuals who had consumed nothing by mouth during the previous 12-36 h, it seems

possible that these species could be related to natural components of gastric juice. In a recent study (Kyrtopoulos *et al.*, 1985b), we observed that, following in-vitro treatment of fasting gastric juice from individuals with a similar background to those studied here, the concentration of 'total *N*-nitroso compounds', as measured by the method of Walters *et al.* (1978), reached a maximum value of about 8  $\mu$ M. The concentrations of *O*<sup>6</sup>-etdGuo and *O*<sup>6</sup>-medGuo observed in the present study were  $\leq$  1.3 nM and 110 nM, respectively. While it is premature to attribute with certainty the alkylating activity observed to *N*-nitroso compounds, the levels of such activity observed are not incompatible with this possibility.

### Competition of gastric juice-treated DNA with <sup>3</sup>H-methylated DNA for repair by rat-liver AAT

The properties of (1) a relatively wide spectrum of substrate (*O*<sup>6</sup>-alkylguanine) specificity (Pegg *et al.*, 1984) and (2) suicide repair of rat liver AAT were utilized in a competitive repair assay, in an attempt to detect *O*<sup>6</sup>-alkylguanine-type damage in gastric juice-treated DNA. In this assay, following the incubation for 2 h of a rat-liver extract with gastric juice-treated DNA, <sup>3</sup>H-methylated DNA was added and the repair of *O*<sup>6</sup>-[<sup>3</sup>H]methylguanine was followed for 5, 10 or 60 min. The kinetics of this repair were compared to those observed in the presence of DNA not previously treated with gastric juice (see caption to Figure 2).

Fig. 2. Inhibition of repair of *O*<sup>6</sup>-[<sup>3</sup>H]methylguanine (*O*<sup>6</sup>-[<sup>3</sup>H]meG) in <sup>3</sup>H-methylated DNA by gastric juice-treated DNA



Cal-thymus DNA (2.5 mg/ml) was treated with nitrite-enriched gastric juice, as described for 2'-deoxyguanosine (see caption to Fig. 1). After this treatment, the DNA was purified by two phenol-chloroform extractions, one RNase treatment followed by a further phenol-chloroform extraction and finally precipitated with *n*-propanol. For the competitive repair assay, 1 mg DNA was incubated for 2 h at 37°C with rat-liver extract containing about 1 pmol *O*<sup>6</sup>-methylguanine-DNA methyltransferase activity, and then [<sup>3</sup>H]-methylated DNA, containing 1.4 pmol *O*<sup>6</sup>-[<sup>3</sup>H]methylguanine was added and incubation continued for 0-60 min. The amount of *O*<sup>6</sup>-[<sup>3</sup>H]methylguanine removed was estimated by acid hydrolysis-high-performance liquid chromatography. A, DNA treated with methylating, ethylating and butylating agents; B, DNA treated with eight different samples of gastric juice. MNNG, *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine; ENNG, *N*-ethyl-*N*'-nitro-*N*-nitrosoguanidine; ENU, *N*-ethyl-*N*-nitrosourea; BNNG, *N*-butyl-*N*'-nitro-*N*-nitrosoguanidine

Figure 2A shows that DNA previously treated with methylating, ethylating or butylating agents can reduce the rate and/or overall extent of repair of the  $^3\text{H}$ -methylated substrate. When DNA treated with eight different samples of gastric juice was utilized in this assay, while in no case was any significant reduction in the overall extent of  $O^6$ - $^3\text{H}$  methylguanine repair observed (Fig. 2B), in four cases a decrease in the rate of this repair was noted. This observation, while indicating absence of detectable damage capable of undergoing suicide repair by AAT, is compatible with the presence of damage (probably  $O^6$ -alkylguanine-type) capable of acting as a competitive inhibitor of the enzyme, i.e., capable of recognition by and binding to the AAT but not of overall removal. It should be noted that when 2'-deoxyguanosine was exposed under similar conditions to gastric juice (see above), the extents of  $O^6$ -alkylation observed were such that, if present in DNA, they would correspond to 0-162 fmol/mg DNA, a range of levels not expected to be detectable in the assay used here.

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# INTRAGASTRIC NITROSATION AND PRECANCEROUS LESIONS OF THE GASTROINTESTINAL TRACT: TESTING OF AN ETIOLOGICAL HYPOTHESIS

M. Crespi<sup>1</sup>, H. Ohshima<sup>2</sup>, V. Ramazzotti<sup>1</sup>, N. Muffoz<sup>2</sup>, A. Grassi<sup>1</sup>, V. Casale<sup>1</sup>, H. Leclerc<sup>3</sup>, S. Calmels<sup>2</sup>, C. Cattoen<sup>3</sup>, J. Kaldor<sup>2</sup> & H. Bartsch<sup>2</sup>

<sup>1</sup>National Cancer Institute 'Regina Elena', Viale Regina Elena 291, 00161 Rome, Italy; <sup>2</sup>International Agency for Research on Cancer, 150 cours Albert-Thomas, 69372 Lyon, France; and <sup>3</sup>INSERM, U 146, Domaine du CERTIA, 369 rue Jules-Guesde, 59650 Villeneuve d'Ascq, France

The *N*-nitrosoproline (NPRO) test was used to study whether subjects with precancerous conditions of the stomach have an elevated potential for endogenous nitrosation. The highest yield of NPRO after ingestion of beetroot juice (as a source of nitrate) and proline was seen in subjects whose pH of fasting gastric juice was about 1.5-2. No increased level of NPRO was detected in subjects with more advanced lesions, compared to those with a normal stomach. Counts of total and nitrate-reducing bacteria were positively correlated with the pH of gastric juice but did not correlate with the urinary level of NPRO in the same individuals. Bacteria and intragastric nitrosation are discussed as possible etiological factors in human stomach cancer.

We are currently using the NPRO test (Bartsch *et al.*, 1983a; Ohshima *et al.*, 1985) in collaborative clinical studies of subjects at high risk of developing stomach cancer, such as patients with chronic atrophic gastritis (CAG) and pernicious anaemia and those who have undergone gastric surgery like Billroth II gastrectomy, in order to test the etiological association between intragastric nitrosation and stomach cancer (Bartsch *et al.*, 1984). It has been postulated that the achlorhydric stomach found in such patients may provide a suitable milieu for intragastric formation of *N*-nitroso compounds owing to the presence of large numbers of bacteria, which may be involved in the conversion of nitrate to nitrite and subsequent nitrosation *in vivo* (Correa *et al.*, 1975; Reed *et al.*, 1981a; Charnley *et al.*, 1982). Urinary levels of NPRO are being determined as an index of endogenous nitrosation in these patients, and some interim results on subjects with CAG have already been reported (Bartsch *et al.*, 1984). The present paper deals with the relationships between NPRO levels, type and count of bacteria identified in gastric juice samples and the presence of gastric lesions in these subjects.

## Study subjects and procedures

The study subjects and procedures were described in detail in a previous report by Bartsch *et al.* (1984). The procedures included (1) completion of a questionnaire, (2) gastroscopy, (3) collection of fasting gastric juice, (4) collection of biopsies and (5) collection of 24-h urine for the NPRO test. The subjects in the present studies were classified into three groups according to the results of histological evaluations of biopsy samples, as shown in Table 1: Group I (n=15), subjects with normal stomach mucosa or with superficial gastritis only; Group II (n=17), those with mild CAG, with or without mild intestinal metaplasia; Group III (n=18), those with moderate and severe CAG with or without intestinal metaplasia and those with dysplasia. Characteristics of study subjects are given in Table 1.

**Table 1. Characteristics of study subjects, histological evaluation, pH of fasting gastric juice and bacterial counts in the stomach<sup>a</sup>**

	Group I <sup>b</sup>	Group II <sup>c</sup>	Group III <sup>d</sup>
No. of subjects			
Male	9	8	9
Female	7	9	9
Total	16	17	18
Median age (years)	51	48	52
(range)	(33-69)	(35-70)	(29-76)
Histological evaluation (no. of subjects)			
N	4	0	0
SG	12	0	0
mild CAG + SG	0	9	0
mild CAG + mild IM	0	8	0
severe CAG	0	0	2
severe CAG + severe IM	0	0	13
DYS	0	0	3
Smoking habit (no. of subjects)			
nonsmoker	10	10	13
smoker	6	7	5
Median pH of fasting gastric juice (range)	1.75 (1.2-7.0)	1.90 (1.0-7.6)	2.85 (1.0-7.9)
Total bacteria (count/ml)			
median	3800	$5.4 \times 10^4$	$4.5 \times 10^4$
range	$(0-2.1 \times 10^7)$	$(0-4.2 \times 10^7)$	$(0-5.2 \times 10^7)$
Nitrate-reducing bacteria (count/ml)			
median	0	225	800
range	$(0-10^5)$	$(0-6 \times 10^6)$	$(0-1.9 \times 10^6)$

<sup>a</sup>N, normal mucosa; SG, superficial gastritis; CAG, chronic atrophic gastritis; IM, intestinal metaplasia; DYS, dysplasia

<sup>b</sup>Subjects with normal stomach or superficial gastritis

<sup>c</sup>Subjects with mild CAG with or without IM

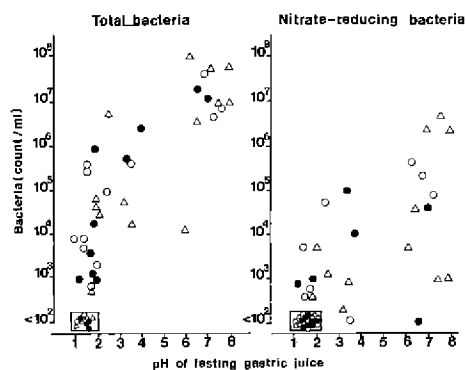
<sup>d</sup>Subjects with moderate or severe CAG with or without IM and DYS

### Relationship between pH of fasting gastric juice and bacteria identified in the stomach

Table 1 shows the pH of fasting gastric juice and counts of total and nitrate-reducing bacteria in the gastric juice. In accordance with the results of other studies (Drasar *et al.*, 1969; Reed *et al.*, 1981a; De Bernadinis *et al.*, 1983; Mueller *et al.*, 1983, 1984; Kyrtopoulos *et al.*, 1985a), a positive correlation was observed between pH of fasting gastric juice and counts for both total and nitrate-reducing bacteria (Fig. 1). In addition, there was a tendency that subjects with more advanced gastric lesions had increased pH values and

more bacteria in their gastric juice (Table 1), although the differences were not statistically significant. It is noteworthy that wide ranges of pH (from 1.0 to 7.9) and bacterial counts (from 0 to  $5.2 \times 10^7$ /ml) were observed in each of the three subject groups.

**Fig. 1 Relationship of gastric bacterial count to pH**



○, Group I (normal or superficial gastritis); ●, Group II (mild CAG with or without intestinal metaplasia); △, Group III (moderate or severe CAG with or without intestinal metaplasia and dysplasia)

Bacterial strains identified in the gastric juices are summarized in Table 2. The samples of Group-III subjects, especially those with higher pH values ( $\text{pH} > 4$ ), were more frequently found to contain total *Enterobacteria*, glucose non-fermentative bacteria, *Haemophilus influenzae*, *Neisseria* and *Streptococcus viridans*. Gastric juice with an acidic pH ( $< 3.9$ ) was normally sterile, although some samples contained *Lactobacillus* and *Enterobacteria*. Thirty microorganisms isolated from these gastric juice samples were examined for their capacity to nitrosate morpholine at pH 7.2 and to reduce nitrate; nine strains were found to catalyse the in-vitro nitrosation of morpholine with a specific nitrosation activity ranging from 1 to 308 nmol *N*-nitrosomorpholine/mg protein per h, and 22 strains exhibited a wide range of nitrate-reducing activities (Calmels *et al.*, this volume).

### Relationship between the urinary level of *N*-nitrosamino acids, bacterial count and pH of the gastric juice

The levels of NPRO and other *N*-nitrosamino acids [*N*-nitrosothiazolidine 4-carboxylic acid (NTCA), *N*-nitroso-2-methylthiazolidine 4-carboxylic acid (NMTCA), *N*-nitroso-sarcosine (NSAR)] in the urine of the subjects who had ingested 260 mg nitrate in beetroot juice and then 500 mg L-proline, were analysed as an index of endogenous nitrosation (Bartsch *et al.*, 1983a, 1984; Ohshima *et al.*, 1985). The urinary levels of the three nitrosamino acids and their sum in the three study groups are given in Table 3, where data are given separately for all subjects (pH 1-8) and for those with pH 1-3. These results are also plotted against the pH of the gastric juice from the same individuals (Fig. 2). The NPRO level in the urine ranged from trace amounts to  $108 \mu\text{g/day}$  per person. As noted previously (Bartsch *et al.*, 1984), the level appeared to be dependent on the pH of the gastric juice, as the highest values for NPRO and other nitrosamino acids were seen at pH 1.5-2. Subjects with a gastric pH of 6-8 excreted relatively low levels of NPRO. Group-III subjects with the most advanced lesions excreted smaller amounts of nitrosamino acids in their urine, compared to those of the two other groups ( $p < 0.05$ ). Smokers tended to excrete higher levels of nitrosamino acids than nonsmokers. As shown in Figure 3, no apparent correlation was observed between the urinary level of either NPRO or the sum of the nitrosamino acids and the count of total and nitrate-reducing bacteria in gastric juice.



**Table 2. Bacterial strains and frequencies of isolation in gastric juice with low pH (< 3.9) and high pH (> 4.0)<sup>a</sup>**

	Group I (N, SG)		Group II (mild CAG ± IM)		Group III (severe CAG, ± IM, DYS)	
	pH < 3.9	pH > 4	pH < 3.9	pH > 4	pH < 3.9	pH > 4
No. of samples tested	12	3	11	3	11	7
Total bacterial count > 10 <sup>4</sup>	4	3	4	3	6	7
Nitrate-reducing bacteria > 10 <sup>3</sup>	3	2	2	3	2	6
<b>Aerobic bacteria:</b>						
(+) Total enterobacteria > 10 <sup>2</sup>	3	1	1	2	2	5
(+) <i>Escherichia coli</i>	1	0	0	0	1	2
(+) <i>Enterobacter cloacae</i>	1	1	0	1	0	3
(+) <i>Proteus hauseri</i>	2	0	0	2	1	0
(+) <i>P. morgani</i>	1	0	0	0	0	0
Glucose non-fermentative bacteria > 10 <sup>2</sup>	1	1	0	2	1	5
(-) <i>Pseudomonas maltophilia</i>	1	1	0	2	1	5
(±) <i>Flavobacterium</i>	0	1	0	1	0	2
Unidentified microorganisms	1	1	0	1	1	3
(+) <i>Haemophilus influenzae</i> > 10 <sup>2</sup>	0	0	0	2	0	5
(+) <i>Neisseria</i> > 10 <sup>2</sup>	1	3	1	3	2	4
(+) <i>N. mucosa</i>	0	1	1	3	2	4
(+) <i>N. perflava</i> 1	0	0	2	1	1	4
Unidentified microorganisms	0	0	0	0	1	1
(+) <i>Saprophyte corynebacteria</i> > 10 <sup>2</sup>	0	0	1	1	3	2
(-) <i>Lactobacillus</i> > 10 <sup>2</sup>	6	3	7	2	7	4
(±) <i>Staphylococcus aureus</i> > 10 <sup>2</sup>	0	1	0	1	0	1
(-) <i>Streptococcus viridans</i> > 10 <sup>2</sup>	3	3	1	3	3	7
Others	4	0	3	0	1	0
<b>Anaerobic bacteria: &gt; 10<sup>2</sup></b>						
(±) <i>Clostridium perfringens</i>	1	0	1	0	0	0
(-) <i>Bifidobacterium bifidum</i>	0	1	0	0	0	1
(±) <i>Bacillus</i> gram positive	0	0	0	1	1	0
Yeast: > 10 <sup>2</sup>	2	0	2	1	1	1

<sup>a</sup>No. of samples given; abbreviations as in legend to Table 1; nitrate reductase activities: (+), positive; (-), negative; (±), positive/negative

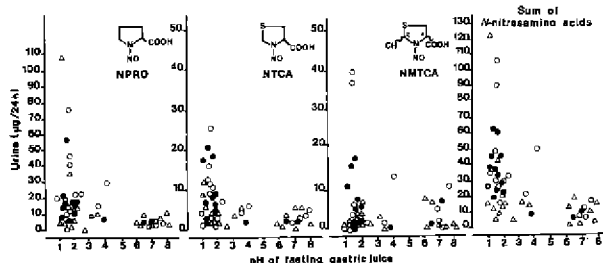
**Table 3. Urinary excretion of *N*-nitrosamino acids in study subjects**

Subject group <sup>d</sup>	No. of subjects	pH	<i>N</i> -Nitrosamino acid (µg/person per day; median and 95% confidence interval)			
			NPRO	NTCA	NMTCA	Sum
Group I	15	1-8	14.4(6.4-17.9)	6.1(3.6-9.4)	1.1(0.5-4.2)	27.2(14.9-36.6)
Group II	17		12.1(4.5-20.4)	4.0(2.6-7.6)	2.3(0.7-6.1)	24.5(13.7-34.8)
Group III	18		6.3(2.9-9.3)	3.4(1.9-4.1)	1.0(0.5-2.1)	13.9(6.1-16.1)*
Group I	12	1-3.9	15.8(10.3-26.0)	6.9(3.6-11.2)	3.3(0.5-7.2)	33.7(22.0-42.5)
Group II	12		17.3(9.5-27.9)	5.9(2.9-10.8)	1.8(0.3-7.2)	28.4(19.5-49.4)
Group III	11		9.0(4.2-21.5)	3.5(2.6-6.2)	1.3(0.4-2.7)	15.0(10.2-32.8)
Smoker	18		13.3(4.9-25.5)	4.3(2.4-6.9)	2.4(0.7-5.6)	25.9(11.6-44.3)
Nonsmoker	32		8.7(4.9-10.9)	4.1(2.9-5.4)	1.2(0.6-2.4)	15.7(12.4-20.7)
All subjects combined	50		9.3(5.7-12.2)	4.1(3.1-5.2)	1.5(0.9-2.5)	17.8(13.9-23.6)

<sup>d</sup>For explanation, see legend to Table 1

\* Significantly different from Groups I and II (*p* < 0.05)

**Fig. 2. Urinary excretion of *N*-nitrosamino acids by subjects after ingestion of beetroot juice and proline versus pH of fasting gastric juice**



●, Group I; ○, Group II; △, Group III (for details see legend to Fig. 1)

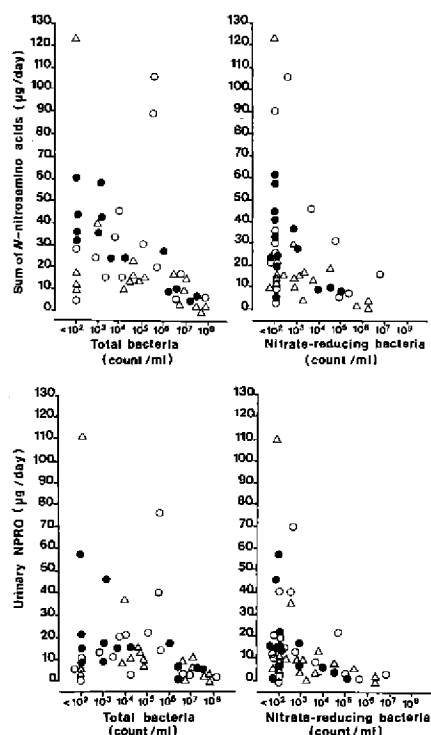
findings are in contrast to those of other reports that higher levels of bacteria, nitrite and total *N*-nitroso compounds were detected in the stomach of such patients (Correa *et al.*, 1975; Schlag *et al.*, 1980; Reed *et al.*, 1981a). The reason for this discrepancy could be due to the following:

(1) Although bacteria isolated from humans catalyse nitrosation of a wide range of secondary amines, the nitrosation activity of proline has been shown to be rather low

**Nitrosation in the achlorhydric stomach and the NPRO test**

In the present study, the highest yield of NPRO was seen in subjects whose gastric pH was about 1.5-2.0. Under such conditions, gastric juice is normally sterile, and, therefore, no correlation was observed between bacterial counts and urinary NPRO level. Thus, our data do not support the notion that the formation of *N*-nitroso compounds is favoured in the achlorhydric stomach of CAG patients, with increased bacteria colonization. These

**Fig. 3. Relationship of urinary NPRO and sum of *N*-nitrosamino acids with bacterial count detected in gastric juice**



○, Group I; ●, Group II; △, Group III (for details see legend to Fig. 1)

concentration of nitrite is formed by bacteria in the intestinalized areas and the lowest pH is provided by gastric acid secreted by normal mucosa (Charney *et al.*, 1982).

(4) In a model of nitrosation in the achlorhydric stomach (Correa *et al.*, 1975), increased nitrosamine formation was postulated as a consequence of gastric lesions such as intestinal metaplasia. However, a well-known gastric carcinogen, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and its analogues have been shown to induce intestinal metaplasia in the stomachs of experimental animals (Sugimura *et al.*, 1982). This suggests that *N*-nitroso compounds formed intragastrically in early life could play an important role in inducing gastric lesions. More work is warranted to identify the *N*-nitroso compounds formed in the normal stomach and to study their biological effects, as well as the origin of the precursor amines. Bacterial colonization has recently been shown to increase the incidence of gastric tumours induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in experimental animals (Sumi & Miyakawa, 1981; Morishita & Shimizu, 1983); bacteria may produce agents which act as

compared to that for nitrosation of morpholine (Calmels *et al.*, 1985). Thus, bacteria present in the stomach did not catalyse intragastric nitrosation of proline, resulting in a low yield of NPRO in the urine.

(2) Chemical nitrosation under the acidic conditions of the normal stomach may be kinetically more relevant than bacteria-catalysed nitrosation in the achlorhydric stomach. Although nitrite concentrations are elevated in the achlorhydric stomach, the pH of the gastric environment is increased owing to the reduction of gastric acid secretion. Under conditions of neutral or basic pH, chemical nitrosation occurs much more slowly than under acidic conditions. In normal subjects, although gastric nitrite is not detectable, a considerable amount of nitrite is generated by bacterial reduction of nitrate in the oral cavity, and this may react much more rapidly with amino compounds present in the acidic gastric juice. Thus, the balance between the concentration of nitrite and acid-catalysed nitrosation is an important rate-determining step for intragastric nitrosation. In this respect, more kinetic studies on nitrosation will be needed.

(3) The NPRO test may not be a suitable procedure to reveal localized nitrosation occurring at the border of the normal and metaplastic areas of the stomach. This border has been suggested to be the most favourable site for chemical nitrosation, because the highest

cocarcinogens or promote tumorigenesis. A specific type of bacteria, *Campylobacter pyloridis*, recently identified in the human stomach mucosa as a possible etiological agent of gastritis (Hazell & Lee; 1986), may also contribute to gastric carcinogenesis by inducing chronic irritation or inflammation.

**Acknowledgements**

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# THE AVAILABILITY OF DIETARY NITRATE FOR THE ENDOGENOUS NITROSATION OF L-PROLINE

T.M. Knight<sup>1</sup> & D. Forman

*Imperial Cancer Research Fund, Radcliffe Infirmary, Oxford, UK*

Urinary excretion of *N*-nitrosoproline (NPRO) following ingestion of a high-nitrate salad meal, without then with an oral dose of 500 mg L-proline, was investigated in 16 healthy volunteers. The mean excretion rate following consumption of the high-nitrate meal alone was significantly lower than that measured after ingestion of the salad plus proline. Supplementation of the same meal (plus proline) with vitamin C from dietary sources resulted in a significant decrease in mean urinary NPRO levels in healthy subjects. The nitrosation-inhibiting effect of vitamin C was not affected by an increase in the fat content of the meal. Supplementation of the high-nitrate salad with alcohol or coffee did not affect subsequent urinary NPRO levels. No significant difference was observed in the urinary NPRO concentrations of smokers and nonsmokers after ingestion of high-nitrate salad (with or without vitamin C) plus proline.

Ingestion of nitrate and/or proline can lead to the endogenous formation of NPRO, a reaction that can be inhibited by concurrent intake of other dietary components, such as vitamin C. The techniques developed for the assessment of individual ability to form NPRO are applied here to assess whether subjects consuming controlled but nutritionally normal sources of reactants show the same effects.

## Study subjects

Sixteen volunteers, aged 23-56 years, of each sex, participated in the first study. The second study involved 19 volunteers, all women aged 20-28 years.

## Test meals

The basic test meal (meal one) consisted of a salad designed to provide large but not abnormal amounts of nitrate and low levels of vitamins C and E and fat. Triplicate samples of this meal (frozen to  $-40^{\circ}\text{C}$  immediately after preparation and subsequently analysed by Dr C.L. Walters, British Food Manufacturers Industrial Research Association, Leatherhead, Surrey, UK) were found to contain, on average, 168.5 mg nitrate (range, 164.5-172.5), with nitrite and NPRO levels below the limits of detection. Approximate levels of vitamins C and E and fat were estimated to be 25 mg, 0.5 mg and 20 g, respectively, on the basis of the British composition of food tables (Paul & Southgate, 1978).

In the second study, four variations of the basic meal were introduced. Meal two consisted of the same ingredients as used in meal one above, with the addition of foods rich in vitamin C (raw green pepper, fresh strawberries and blackcurrant health drink) to increase the vitamin C content of the meal to an estimated 325 mg. For meal three, the high

<sup>1</sup> To whom correspondence should be addressed

vitamin C formula was repeated but the estimated fat content of the meal was increased from 20 to 95 g by supplementation of low-fat cheese and salad dressing with high-fat alternatives, the addition of double cream to the fruit and a doubling of the butter ration. Meals four and five repeated meal one, but with the consumption of 225 ml white wine (supplying an estimated 20 g ethanol) or two cups of coffee (made with 5 g ground coffee) as a source of polyphenolic compounds such as caffeine and chlorogenic acid.

Foods for both studies were purchased daily and prepared no more than 2 h before consumption. The additional foods used in the second study have not been reported to contain substantial amounts of nitrate, nitrite or preformed NPRO.

### Dietary restrictions

In order to minimize the confounding effect of dietary (preformed) NPRO (Stich *et al.*, 1984a) and modifiers of nitrosation, subjects were not permitted to consume cured meats, smoked fish, beers, whisky or vitamin supplements for a period starting 72 h prior to each test meal and continuing until the end of the 24-h urine collecting period (25 h after the end of the test meal). Consumption of any form of alcohol was not allowed during urine collections. Subjects fasted for at least 2 h after the end of the test meals, and dietary records (semiquantitative) were kept for the 25 h following the test meals. Apart from these restrictions, subjects were instructed to follow as normal a life as possible. Smoking habits were not curtailed, but smokers were requested to keep habits constant, i.e., to smoke similar amounts at similar times for the 25-h period following test meals. The number and times of cigarettes smoked during this period were recorded.

### Study design

*Study one:* The aim of this study was to compare NPRO excretion following the basic test meal, with and without a proline loading dose. Subjects consumed test meal one in week one of the study, and began a 24-h urine collection 1 h after the end of the meal. One week later (week two), the same procedure was repeated with the addition of an oral dose of L-proline (500 mg dissolved in 20 ml drinking-water), taken a few minutes before the start of the 24-h urine collection.

*Study two:* The aim of this study was to compare NPRO excretion following the basic test meal with a proline loading dose, with and without additional dietary vitamin C (for all subjects) and subsequently with either vitamin C/high fat (six subjects) or alcohol (five subjects) or polyphenol (four subjects) supplementation. Thus, three test meals were consumed (one per week), each followed, after 1 h, by ingestion of the proline dose and the start of a 24-h urine collection. Saliva samples were also collected, 10 min before each test meal and again immediately before each proline dose. Test meal one was consumed in week one, meal two in week two, and in the third study week subjects chose to eat one of meals three, four or five.

All subjects were fully familiar with the aims of the studies and were given detailed instructions for the correct collection of 24-h urine samples. None reported any illness or were taking any medicines likely to affect the outcome of the studies.

### Sample collection and analysis

Urine samples were collected and analysed following published methods (Ohshima & Bartsch, 1981; Ohshima *et al.*, 1982, 1984b) by Dr H. Ohshima (IARC, Lyon, France).

In study two, estimations of exposure to dietary nitrate and nitrite were made by measuring these ions in the saliva and in 24-h urine samples, using the technique developed by Phizackerley and Al-Dabbagh (1983).

### Endogenous formation of NPRO

NPRO excretion in 24-h urines of subjects consuming test meal one was significantly greater following the L-proline loading dose (Table 1). Mean concentrations of the other *N*-nitroso compounds detected in the urine samples (*N*-nitrosothiazolidine 4-carboxylic acid, *N*-nitroso(2-methylthiazolidine) 4-carboxylic acid, *N*-nitrososarcosine, *N*-nitroso-propylmethylamine and *N*-nitrosoazetidine carboxylic acid) did not differ significantly as a result of the proline dose. Apart from NPRO, only *N*-nitrosothiazolidine 4-carboxylic acid and *N*-nitroso-propylmethylamine were present in a majority of urine samples, with mean concentrations of 2.50 and 1.00  $\mu\text{g}/24\text{ h}$  (week one), and 3.60 and 0.90  $\mu\text{g}/24\text{ h}$  (week two), respectively.

**Table 1. Urinary excretion of NPRO ( $\mu\text{g}/24\text{ h}$ ) following consumption of different test meals**

Test meal	N	Geometric mean (95% confidence interval)	<i>p</i> value <sup>a</sup>
<b>Study one</b>			
High nitrate meal	16	2.7 (2.2-3.3)	
High nitrate + proline	16	10.8 (7.9-14.9)	<0.001
<b>Study two</b>			
High nitrate + proline	19	28.4 (17.3-46.8)	
High nitrate/vitamin C + proline	19	15.8 (11.2-22.5)	<0.05
High nitrate/vitamin C + proline	6	12.3 (6.3-24.3)	NS
High nitrate/vitamin C + fat + proline	6	8.9 (3.7-21.1)	
High nitrate + proline	5	36.5 (20.4-65.4)	NS
High nitrate + wine + proline	5	33.1 (12.0-91.8)	
High nitrate + proline	4	40.9 (17.3-96.8)	NS
High nitrate + coffee + proline	4	35.6 (11.9-106.2)	

<sup>a</sup>From matched pairs *t*-test; NS, not significant

After consumption of the high-nitrate meal plus proline, mean conversion rates of nitrate and proline to NPRO were 0.0025% and 0.0016%. In the present study, we observed wide individual variation in ability to form NPRO from the given precursors, with conversion rates ranging from 0.0004% to 0.006% for nitrate and 0.0002% to 0.004% for proline. These rates were significantly higher in women ( $p < 0.05$ ). Smoking, previously demonstrated to raise urinary NPRO levels in some studies (Hoffmann & Brunne-mann, 1983; Brunne-mann *et al.*, 1984; Ladd *et al.*, 1984b; Tsuda *et al.*, 1986), could not be the reason for these differences, as the female subjects were all nonsmokers. There was only one male smoker in this study and removal of his data

from the analyses does not alter the mean conversion rates for males. Age (testing for less than 40 years against 40+ years) did not significantly affect conversion rates.

The wide range in individual nitrosating abilities is further demonstrated by the results of study two (Table 1), where, in week one, an even greater range was seen than in the comparable data from study one. The mean urinary NPRO level measured following the vitamin C-rich test meal two is significantly lower than that after test meal one.

It is interesting that subjects showing relatively high urinary NPRO levels after test meal one (study two) had, on average, greater reductions following the high vitamin C meal. Taking an arbitrary cut-off point of 20  $\mu\text{g}/24\text{ h}$ , for those with NPRO levels over 20  $\mu\text{g}$ , the difference between NPRO levels following meals one and two is significant ( $p = 0.005$ ), with geometric means of 47.5 and 19.3  $\mu\text{g}/24\text{ h}$ , respectively; whereas for those in the lower group ( $< 20\ \mu\text{g}$  for meal one), there is no difference at all (geometric mean levels, 9.3 and 10.4  $\mu\text{g}/24\text{ h}$ , respectively).

Rates of conversion of proline and nitrate to NPRO for test meal one of study two are of the same order of magnitude as those calculated for week two of the first study: 0.008% (proline) and 0.012% (nitrate). Corresponding results for test-meal two (study two) are 0.003% and 0.005%.

Inclusion of high-fat foods with the high vitamin C test meal (meal three) did not significantly alter the mean level of urinary NPRO excreted.

Consumption of alcohol or coffee with the basic high-nitrate meal (test meals four and five) did not result in mean urinary NPRO concentrations significantly different from those obtained in week one for the same subjects.

Five subjects in study two smoked cigarettes (no more than two) between the end of the test meals and proline ingestion, and others during the rest of the experimental period (totals range from 8 to 17). For test meal one, mean NPRO levels ( $\mu\text{g}/24\text{ h}$ ) were 39.4 for smokers and 25.3 for nonsmokers ( $p = 0.53$ ). Both subgroups demonstrated the same percentage difference between NPRO levels in weeks one and two ( $-10.3\%$ ). No significant difference was observed between smokers and nonsmokers for the sum total of all five *N*-nitroso compounds for week one or two.

*N*-Nitrosothiazolidine 4-carboxylic acid and *N*-nitrososarcosine were also detected in urine samples in study two in relatively large amounts, but mean concentrations did not differ significantly between tests. The other *N*-nitroso compounds detected occurred only sporadically at low concentrations in urines from both studies.

Levels of nitrate and nitrite in saliva and nitrate in urine did not vary significantly between study weeks, hence mean values are given in Table 2. These data show highly significant increases in salivary concentrations of both ions after consumption of the high-nitrate foods. Levels of nitrate and nitrite did not differ significantly (in urine and saliva either before or after meals) between smokers and nonsmokers.

The extent to which food-bound amines, amides and other substrates, as opposed to free amino acid, may be nitrosated by food nitrate is unclear. Our test meal one contained approximately 2.0 g of proteinaceous proline, yet urinary NPRO levels following consumption of this meal resemble the low background concentrations observed in other studies (Ohshima & Bartsch, 1981; Ohshima *et al.*, 1984b; Wagner *et al.*, 1985; Lu *et al.*, 1986) and increase significantly when only 0.5 g of free proline are ingested.

The concentrations of urinary *N*-nitrosothiazolidine 4-carboxylic acid and *N*-nitroso-(2-methylthiazolidine) 4-carboxylic acid measured in the present study are comparable to those reported in other studies. It has been suggested that urinary concentrations of these and other *N*-nitrosamino acids may be used collectively as indicators of exposure to *N*-nitroso compounds from exogenous and endogenous sources (Ohshima *et al.*, 1984b; Wagner *et al.*, 1984a, 1985; Ohshima *et al.*, 1986; Tsuda *et al.*, 1986).



**Table 2. Results from study two — urinary nitrate and salivary nitrate and nitrite**

Parameter	N	Geometric mean <sup>a</sup> (95% confidence interval)	p value <sup>b</sup>
Urinary nitrate (mg/24 h)	19	140.6 (117.1-168.8)	
Salivary nitrate (nmol/ml)			
Before meal	19	388.3 (280.0-538.8)	
After meal	19	2078.3 (1816.8-2376.8)	<0.001
Salivary nitrite (nmol/ml)			
Before meal	19	139.0 (102.8-187.8)	
After meal	19	801.1 (682.7-940.2)	<0.001

<sup>a</sup>Geometric mean of data from the three study weeks

<sup>b</sup>From matched pairs *t*-test

The mean rates of conversion of nitrate and proline to NPRO calculated from our results are similar to those observed in the original NPRO-test study by Ohshima and Bartsch (1981): 0.002% (nitrate) and 0.004% (proline).

In other studies of endogenous nitrosation, significant inhibition of NPRO formation has been achieved by ingestion of vitamin C, usually administered in large pharmacological doses, whereas our meals (study two) provided smaller, more 'normal' amounts, in a complex food matrix (Ohshima & Bartsch, 1981; Hoffmann & Brunnemann, 1983; Brunnemann *et al.*, 1984; *et al.*,

1984a; Ohshima *et al.*, 1985; Wagner *et al.*, 1985; Lu *et al.*, 1986). Our results therefore suggest that nitrate contained within certain vegetables may be unavailable for nitrosation due to the high levels of vitamin C also present. Eight subjects showed no decrease in NPRO excretion following test meal two. Smoking does not appear to be responsible, and, except for one subject who took the proline dose 20 min late in week one, there is no methodological explanation. For the subgroup who excreted more than 20 µg/24 h after test meal one (study two), the effect of vitamin C is even more noticeable.

It is possible that for the group with concentrations of NPRO in week one less than 20 µg/24 h there is either no endogenous synthesis of NPRO or a very slow excretion rate, whereas for the other group both background and endogenous NPRO are detected and/or they may also have a faster excretion rate. If either of these hypotheses is correct, we would expect a significant decrease in urinary NPRO levels following consumption of vitamin C only in the latter group.

It is difficult to draw any conclusion about the effects of fat on the inhibition of NPRO formation by vitamin C or of alcohol, coffee or smoking on L-proline nitrosation, owing to the small sample sizes involved. However, other studies have demonstrated a significant effect of cigarette smoking on the promotion of nitrosation (Hoffmann & Brunnemann, 1983; Brunnemann *et al.*, 1984; Tsuda *et al.*, 1986). In a study by Ladd *et al.* (1984b), no such significant difference was observed until subjects were given a nitrate challenge (beetroot juice, supplying 325 mg nitrate).

It has been suggested that a lipid medium could inhibit nitrosation (Kurechi & Kikugawa, 1979). Although we found a 28% decrease in mean urinary NPRO concentration following inclusion of high levels of fat in the vitamin C-rich meal, the decrease was not significant.

Our studies demonstrate that food-borne nitrate is available for the nitrosation of an oral dose of L-proline but suggest that protein-bound substrates may not be as readily nitrosated by dietary nitrate. We did achieve a significant lowering in NPRO levels following ingestion of the vitamin C-rich meal, especially in high nitrosators.

There was much individual variation in the extent of NPRO formation. The percentage conversion of ingested nitrate to gastric nitrite may vary, as may the gastric concentrations of modifiers of nitrosation, such as thiocyanate ions. The nitrosation of proline has a pH optimum of 2.5 (Mirvish *et al.*, 1973b); thus, individual variation in gastric acidity will result in the formation of different amounts of NPRO, the rate of excretion of which will not be identical in all subjects.

The study of nitrosating ability in populations with different social and dietary habits may provide information about the complex interaction between the many factors capable of modifying endogenous nitrosation.

#### **Acknowledgements**

We are grateful to our subjects for their careful and enthusiastic cooperation, to Dr H. Ohshima for the analysis of urine samples and to Dr C.L. Walters for the analysis of duplicate meal samples. We also wish to thank Miss C. Bates for secretarial assistance. This research was financed by the Imperial Cancer Research Fund in collaboration with Oxford Polytechnic.

## GASTRIC HYPOACIDITY AS A RISK FACTOR FOR GASTRIC AND OTHER CANCERS

C.P.J. Caygill<sup>1,4</sup>, S.A. Leach<sup>2</sup>, J.S. Kirkham<sup>3</sup>,  
T.C. Northfield<sup>3</sup>, C.N. Hall<sup>3</sup> & M.J. Hill<sup>2</sup>

<sup>1</sup>PHLS — Communicable Diseases Surveillance Centre, 61 Colindale Avenue, London NW9 5HT, UK; <sup>2</sup>PHLS — Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire SP4 0JG; and <sup>3</sup>Norman Tanner Gastroenterology Unit, St James Hospital, Balham, London SW12 8HW, UK

**Evidence is presented and discussed to test the hypothesis that the excess risk of gastric cancer observed in patients with decreased gastric acidity is caused by metabolites of nitrite, possibly *N*-nitroso compounds.**

If *N*-nitroso compounds are responsible for the excess risk of gastric cancer observed in pernicious anaemia (PA) and gastric surgery (GS) patients, then, since it is unlikely that only one *N*-nitroso compound is formed and since some classes of *N*-nitroso compounds are target-organ specific, tumours at other sites would be expected. Therefore, a study of 5018 GS and 600 PA patients was set up to assess their excess risk for gastric and a number of other cancers. The patients studied and the analytical methods used have been described previously (Caygill *et al.*, 1984, 1986). Here, we describe an updated analysis of the PA patients and a summary of the results on the GS patients. The gastric juice from such patients was analysed.

In the PA patients, there was a 1.5-fold excess risk of all cancers ( $p < 0.01$ ), composed of a 4.7-fold excess risk of gastric cancer ( $p < 0.001$ ), a 1.9-fold excess risk of colorectal cancer ( $p < 0.07$ ), a 2.5-fold excess risk of biliary-tract cancer ( $p < 0.07$ ) and no excess of other neoplasms (41 cases observed, 38.3 cases expected). In the GS patients, there was an overall relative risk of gastric cancer: 3.1-fold ( $p < 0.001$ ) in gastric ulcer (GU) patients and 1.2-fold ( $p$  not significant) in duodenal ulcer (DU) patients. When analysed by time after operation, GU patients had a 2.67-fold ( $p < 0.001$ ) relative risk during the first 20 postoperative years and a subsequent 5.45-fold ( $p < 0.001$ ) excess risk compared with 0.43-fold ( $p < 0.01$ ) and 3.70-fold ( $p < 0.001$ ), respectively, in DU patients (Table 1). There was no excess risk of cancer at any other site during the first 20 post-operative years; in the subsequent years, however, there were excess risks of cancers of the colorectum (1.6-fold,  $p < 0.05$ ), biliary tract (8.6-fold,  $p < 0.001$ ), pancreas (3.8-fold,  $p < 0.001$ ) and oesophagus (2.3-fold,  $p < 0.001$ ) as well as of all sites (3.3-fold,  $p < 0.001$ ). Thus, there is clear evidence of an excess risk of cancer at sites other than the stomach. For each site, the excess was greater for GU than for DU patients, and in all cases there was a latency of 20 years after surgery, except in the case of gastric cancer in GU patients (see Table 2); in PA patients, the date of onset of achlorhydria was not determinable and so a latency could not be determined. The excess

<sup>4</sup> To whom correspondence should be addressed

gastric cancer risk was in the progression PA > GS > normal. Within the GS group, the risk was greater in GU than in DU patients; this is of interest because GU patients will have been hypochlorhydric for longer than DU patients.

**Table 1. Gastric cancer mortality by type of ulcer<sup>a</sup>**

Years after operation	DU			GU			Others			Total		
	O	E	O/E	O	E	O/E	O	E	O/E	O	E	O/E
0-4	0	3.9	-	9	2.8	3.21***	1	1.2	0.83	10	7.8	1.28
5-9	3	4.9	0.61	9	3.1	2.90***	1	1.3	0.76	13	14.2	0.92
10-14	2	5.8	0.34*	9	3.2	2.81***	0	1.2	-	11	10.2	1.08
15-19	4	6.2	0.65	5	2.9	1.72	1	1.0	1.00	10	10.1	0.99
0-19	9	20.9	0.43**	32	12.0	2.67***	3	4.7	0.64	44	42.3	1.04
20+	20	5.4	3.70***	12	2.2	5.45***	4	0.8	5.00***	36	8.4	4.39***

<sup>a</sup>DU, duodenal ulcer; GU, gastric ulcer; others, patients who had both GU and DU or who had a stomach ulcer after previous surgery

\*  $p < 0.05$

\*\*  $p < 0.01$

\*\*\*  $p < 0.001$

**Table 2. Mortality from a number of different cancers after gastric surgery for ulcers, by ulcer site**

Cancer	No. of patients	Site of ulcer	0-19 years after operation			20+ years after operation		
			O	E	O/E	O	E	O/E
Gastric	2577	DU	9	20.90	0.4**	20	5.50	3.6***
	1385	GU	32	12.00	2.7***	12	2.20	5.5***
Colorectal	2577	DU	23	28.50	0.8	6	7.95	0.8
	1385	GU	8	17.05	0.5**	11	3.66	3.0**
Biliary-tract	2577	DU	4	1.62	2.5	2	0.39	5.1
	1385	GU	1	1.02	1.0	3	0.19	15.8**
Breast (female only)	438	DU	5	6.62	0.8	5	1.26	4.0**
	442	GU	1	6.57	0.2***	4	1.01	4.0*
Pancreas	2577	DU	7	10.16	0.7	6	2.50	2.4*
	1385	GU	4	5.91	0.7	6	1.04	5.8**
Oesophagus	2577	DU	4	6.53	0.6	3	1.65	1.8
	1385	GU	5	3.67	1.4	2	0.66	3.0
Bladder	2577	DU	6	9.88	0.6	6	2.79	2.2
	1385	GU	3	6.15	0.5	3	1.20	2.5
Lung	2577	DU	70	68.70	1.0	106	53.20	2.0***
	1385	GU	56	45.60	1.2	79	31.23	3.7***
All neoplasms	2577	DU	192	236.65	0.8**	158	61.82	2.6***
	1385	GU	165	142.95	1.2	115	25.42	4.5***

\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; O, observed; E, expected

We have determined the pH and the concentrations of nitrate-reducing bacteria, nitrate and nitrite in gastric juice samples at hourly intervals for 24 h in two control patients with normal gastric acidity, in three GS patients and in four PA patients (Table 3). The nitrite concentration, the proportion of nitrate reduced to nitrite and the number of nitrate-reducing bacteria were in the same progression as the cancer risk. The median count of nitrate-reducing bacteria correlated with the extent of nitrate reduction ( $p < 0.02$ ). These results are consistent with the hypothesis that metabolites of nitrite, possibly *N*-nitroso compounds, are implicated in the excess risk of gastric (and other) cancer in patients with decreased gastric acidity.

**Table 3. Nitrate, nitrite and nitrate-reducing bacteria in gastric juice from nine subjects sampled hourly over 24 h**

Patient	Nitrate-reducing bacteria <sup>a</sup>			Gastric juice nitrate/nitrite <sup>b</sup>			
	Number	Median	Range	Number	NO <sub>3</sub> (μM)	NO <sub>2</sub> (μM)	$\frac{\text{NO}_2}{\text{NO}_3 + \text{NO}_2}$ (%)
Control	21	6.48	ND - 7.70	25	175 ± 84	29 ± 29	14
Control	19	5.98	ND - 7.13	19	168 ± 62	21 ± 20	11
GS	11	6.94	6.34 - 7.86	10	174 ± 82	42 ± 14	20
GS	24	6.88	6.23 - 8.11	27	145 ± 58	46 ± 23	23
GS	16	5.50	4.84 - 7.47	15	203 ± 14	19 ± 7	9
PA	19	6.94	6.26 - 7.91	25	174 ± 53	111 ± 83	34
PA	19	6.95	6.39 - 7.85	22	94 ± 72	72 ± 83	43
PA	12	6.71	6.24 - 7.10	19	196 ± 91	110 ± 59	34
PA	22	7.20	6.16 - 8.26	27	126 ± 91	56 ± 32	30

<sup>a</sup>log number of colony-forming units; ND, not detected

<sup>b</sup>Mean concentration ± SD

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## EVALUATION OF THE NITROSAMINE HYPOTHESIS OF GASTRIC CARCINOGENESIS IN MAN

C.N. Hall<sup>1,4</sup>, D. Darkin<sup>2</sup>, N. Viney<sup>2</sup>, A. Cook<sup>3</sup>, J.S. Kirkham<sup>1</sup>  
& T.C. Northfield<sup>1</sup>

<sup>1</sup>Norman Tanner Gastroenterology Unit, St James' Hospital, Balham,  
London SW12 8HW; <sup>2</sup>Smith, Kline & French Research Institute,  
Welwyn Garden City, Herts; and <sup>3</sup>PHLS — Centre for Applied Microbiology  
and Research, Salisbury, Wiltshire SP4 0JG, UK

**Patients who had undergone a Billroth II gastrectomy (PG) or had pernicious anaemia (PA) and healthy matched control subjects (MC) participated in 24-h studies in which bacteria, nitrite and *N*-nitroso compounds (NOC) were measured in gastric juice and *N*-nitrosoproline (NPRO) in urine. Consistent with the nitrosamine hypothesis, intragastric levels of bacteria and nitrite were positively related to intragastric pH, but, contrary to the hypothesis, NOC in gastric juice and NPRO in urine were negatively related to intragastric pH.**

The nitrosamine hypothesis of gastric carcinogenesis (Correa *et al.*, 1975) postulates that high intragastric pH promotes the growth of bacteria which reduce dietary nitrate to nitrite and then convert nitrite and dietary nitrogen compounds into carcinogenic NOC. The aim of this study was to test this hypothesis in controlled comparative studies of gastric juice in man.

Three groups of eight to ten PG, PA and MC subjects (matched in triplets for race, sex, age and height) underwent 24-h gastric juice analysis in which intragastric pH, bacteria (total and nitrate-reducing organisms), nitrite and NOC (total and stable) were assessed half-hourly during the day and hourly at night. Details of experimental design and analytical methods are described elsewhere (Milton-Thompson *et al.*, 1982; Keighley *et al.*, 1984). In order to verify the results, the urinary NPRO test of Ohshima and Bartsch (1981) was performed on a separate occasion as an independent parameter of endogenous nitrosation. Creatinine clearance was measured to assess the completeness of urine collection, and NPRO was determined by the method of Sen and Seaman (1984).

### Gastric juice analysis

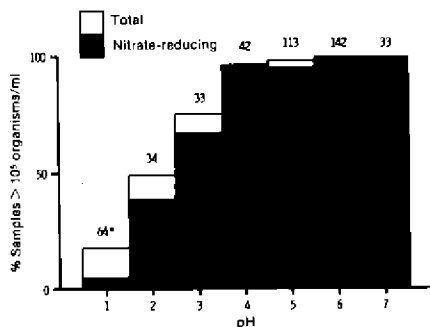
The effect of pH on intragastric factors was assessed by allocating all the samples to different categories of pH, each category encompassing one unit of pH. Bacterial counts of  $> 10^5$  organisms/ml were considered to be pathological, and the percentage number of pathological counts was calculated for each category; mean values were determined for the other factors. Statistical analysis incorporated the paired Student's *t* test for mean data from matched individuals (PG, PA and MC groups) and the Wilcoxon's rank sum test for unmatched data (acidic and hypoacidic groups). The relationships of bacteria, nitrite and

<sup>4</sup> To whom correspondence should be addressed

NOC levels to pH were tested for significance by the chi-squared test of the null hypothesis and by a one-way analysis of variance (Armitage, 1971).

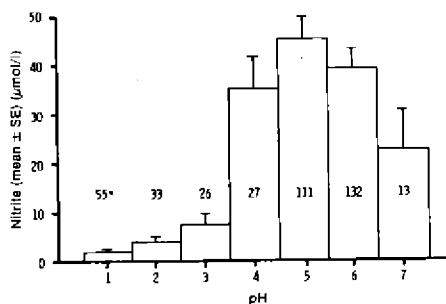
In individual samples, bacteria and nitrite levels increased ( $p < 0.001$ ) but NOC (total and stable) decreased ( $p < 0.001$ ) with increasing intragastric pH (Figs 1-4). Clear differences were not apparent between disease groups because MC and PG were heterogeneous for gastric acidity. Although 8/8 PA were hypoacidic (pH > 4 in > 50% of 24-h samples), only 5/9 PG and 2/9 MC were hypoacidic. When subjects were rearranged into hypoacidic and acidic groups, bacteria and nitrite levels were higher ( $p < 0.01$ ) whereas NOC tended to be lower (not significant) in the hypoacidic group (Table 1).

**Fig. 1. Relationship between incidence of pathological bacterial counts and intragastric pH**

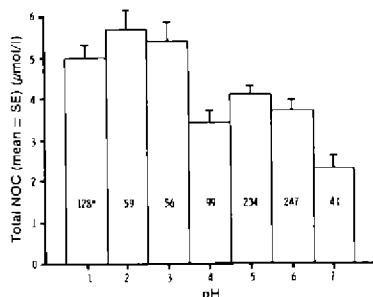


Numbers = samples in each pH category

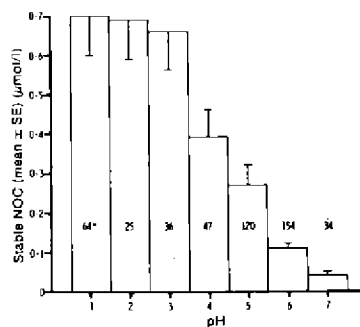
**Fig. 2. Relationship between nitrite and intragastric pH**



**Fig. 3. Relationship between total NOC and intragastric pH**



**Fig. 4. Relationship between stable NOC and intragastric pH**



**Table 1. Analysis of gastric juice in hypoacidic and acidic groups (mean ± SE)**

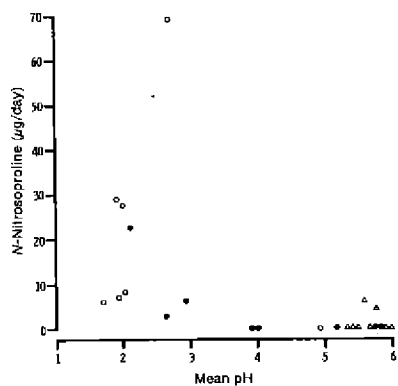
Factor	Acidic	Hypoacidic	<i>p</i> value <sup>a</sup>
Bacteria (% samples with > 10 <sup>5</sup> organisms/ml)			
Total	60 ± 7	100	< 0.01
Nitrate-reducing	53 ± 7	96 ± 2	< 0.01
Nitrite (μmol/l)	11.9 ± 5.0	44.0 ± 7.0	< 0.01
<i>N</i> -Nitroso compounds (μmol/l)			
Total	4.8 ± 0.3	3.4 ± 0.4	NS
Stable	0.52 ± 0.09	0.17 ± 0.02	NS

NS, not significant

**Urine analysis**

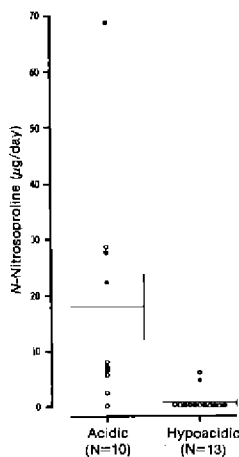
Of the subjects who underwent the NPRO test, 23/29 had previously participated in the gastric juice study. NPRO yields were negatively related to the earlier mean 24-h intragastric pH (*p* < 0.001; Fig. 5). NPRO excretion (mean ± SE) was reduced (*p* < 0.01) in PA (1.1 ± 0.8 ng/day) compared to MC (18.0 ± 7.2 ng/day) and also tended to be lower (not significant) in PG (3.2 ± 2.3 ng/day). Yields were also reduced in hypoacidic compared to acidic subjects (*p* < 0.01; Fig. 6).

**Fig. 5. Relationship of urinary NPRO to intragastric pH**



Δ, PA; ●, PG; ○, MC. Significance tested by Kendall's (1970) rank correlation coefficient

**Fig. 6. Urinary NPRO yields in acidic and hypoacidic groups**



Smokers (●) and nonsmokers (○) were equally represented in each group, as smoking is known to increase yields (Ladd *et al.*, 1984a); creatinine clearance was the same in each group.



Data on bacteria and nitrite in gastric juice are consistent and show positive relationships to intragastric pH, as anticipated in the nitrosamine hypothesis. Data on NOC, however, differ appreciably; some reports indicate increased levels at high pH (Reed *et al.*, 1981a; Stockbrugger *et al.*, 1982), while others found no relationship to intragastric pH (Milton-Thompson *et al.*, 1982; Sturniolo *et al.*, 1983; Bartsch *et al.*, 1984; Keighley *et al.*, 1984). These variable results are largely attributable to the fact that no wholly satisfactory method is available for the determination of NOC in complex biological fluids. In this study, NOC were measured in fresh samples to avoid the possibility of in-vitro storage artefacts, and, additionally, nitrosation was assessed by an alternative, indirect method. The results of these experiments are corroboratory and reveal intragastric pH to be negatively associated with both total and stable NOC in gastric juice and with NPRO in urine. Furthermore, the data on NPRO and intragastric pH correspond closely to those previously reported by Bartsch *et al.* (1984).

In-vitro kinetics under sterile conditions indicate that nitrosation occurs optimally at acid pH (Douglass *et al.*, 1978), and experiments in both gastric juice and saliva (Lane & Bailey, 1973; Tannenbaum *et al.*, 1978) indicate that nitrosation in biological fluids may have characteristics similar to those in the aqueous phase. Our results suggest that chemical mechanisms of nitrosation may predominate *in vivo* in man.

#### Acknowledgements

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# AN INVESTIGATION OF POSSIBLE RISK FACTORS ASSOCIATED WITH GASTRIC CANCER AFTER BENIGN ULCER OPERATIONS

J. Ware<sup>1,5</sup>, G.D. Corcoran<sup>1</sup>, D.W. Day<sup>2</sup>, C.M. O'Donnell<sup>1,3</sup>  
C.J. Newton<sup>4</sup> & W.H. Taylor<sup>4</sup>

<sup>1</sup>Department of Surgery, <sup>2</sup>Department of Pathology, <sup>3</sup> Department of Microbiology,  
University of Liverpool, Royal Liverpool Hospital, Liverpool; and  
<sup>4</sup>Department of Chemical Pathology, Royal Liverpool Hospital,  
Liverpool, UK

The risk of developing gastric cancer has been investigated in a case-control study and in a prospective investigation. In the case-control study, 1495 cases of gastric cancer were identified in five city hospitals and matched with autopsy controls from the same hospitals. The frequency of operations for benign ulcer [partial gastrectomy (PG) and gastroenterostomy] was similar in the two groups. Thus, there was no increased risk for late gastric cancer after an ulcer operation. A total of 140 operated ulcer subjects [80 truncal vagotomy and drainage (TVD), 60 PG and 78 nonoperated cases attending with dyspepsia (C)] were examined by endoscopy, multiple gastric biopsy and analysis of gastric juice for nitrite. Biopsies were graded for gastritis and a gastritis index was derived (normal, 1; superficial gastritis, 2; chronic atrophic gastritis: mild, 3; moderate, 4; severe, 5). More atrophic gastritis was found in operated subjects than in controls: TVD,  $2.3 \pm 0.08$  (mean  $\pm$  SE); PG,  $2.6 \pm 0.1$  versus C,  $1.8 \pm 0.08$ ,  $p < 0.01$ . The severity of atrophic gastritis increased after an operation interval of 20 years in PG subjects ( $p < 0.05$ ). Intestinal metaplasia was a common change, but unequivocal epithelial dysplasia was not observed. Two cases of operated stomach cancer were found. High levels of nitrite were positively correlated with pH and a high gastritis index. This evidence does not suggest that ulcer surgery leads to either an increased risk of cancer or a precancerous condition.

Although the incidence of gastric cancer is decreasing throughout the world, the regional figure for Liverpool (UK) remains relatively high, at 28 per 100 000 population per year. This was an important reason for undertaking the investigations reported. A case-control study to determine the relative risk (RR) of developing gastric cancer more than five years after a benign ulcer operation was first carried out. Cases were 1495 gastric cancers registered in 1970-1979 at five inner city hospitals; they were matched with autopsy controls for hospital source, age, sex and time of death. No case of gastric cancer was included in the control material. The frequency of ulcer surgery was compared in the two groups using matched-pair analysis, and the RR and 95% confidence interval of developing operated stomach cancer were determined using odds ratios (Mantel & Haenzel, 1959). No increased risk was identified after PG, Billroth I or Billroth II, gastroenterostomy alone, or combined (Table 1).

<sup>5</sup> Present address: Department of Surgery, Karolinska Institute, Stockholm, Sweden

**Table 1. Number of operations for benign ulcer disease among cases of gastric cancer (n = 1495) and matched autopsy controls**

Operation	Cases	Controls	RR (95% confidence interval)
Partial gastrectomy	28	22	1.3 (0.7-2.2)
Gastroenterostomy	10	5	2.0 (0.7-5.8)
Total	38	27	1.4 (0.8-2.3)

Confirmation of the validity of the controls was investigated separately. The causes of death among the autopsy controls were compared with data for the region and nationally (Office of Population Censuses and Surveys, 1979). Comparison of the proportional mortality ratios in all three groups confirmed acceptable uniformity (Table 2). A complementary prospective investi-

gation of postoperative stomach was then carried out to compare the prevalence of mucosal changes and gastric juice nitrite in operated and nonoperated subjects.

**Table 2. Proportional mortality ratios (PMR) between autopsies (controls) used in the case-control study and regional and national statistics**

Primary cause of death	Proportional representation			PMR <sup>a</sup> (%)	PMR <sup>b</sup> (%)
	Controls	Regional	National		
Cardiovascular disease	56.2	48.1	50.3	117	113
Malignancy	18.1	20.7	19.9	95	91
Respiratory disease	12.8	16.4	14.5	91	95
General <sup>c</sup>	12.8	12.6	13.3	99	99

Figures based upon decennial grouping. Proportions of four main diagnostic groups given for each group

<sup>a</sup>Control versus regional

<sup>b</sup>Control versus national

<sup>c</sup>Remainder of diagnoses grouped as one

At endoscopy, multiple-paired biopsies and juice samples were obtained; the pH of the juice was recorded and nitrite determined using the Griess reagent. The biopsy sites were standardized, and subsequent examination by one pathologist (DWD) was made 'blind'. Mucosal changes were categorized according to standard criteria (Day, 1986), and a gastritis index derived from scores given to each biopsy site.

Table 3 gives the operative groups and confirms an increased gastritis index in both operated groups compared with nonoperated subjects. Moreover, when the postoperation interval exceeded 20 years, the gastritis index was increased (Table 4). Intestinal metaplasia was a common change, but unequivocal gastric dysplasia was not observed; however, in six cases (one nonoperated) mild dysplasia could not be distinguished from regenerative

changes. Two cases of operated stomach cancer were found. High levels of nitrite were positively correlated (Kendall rank coefficient,  $\tau$ ) with pH ( $\tau = 0.51$ ;  $p < 0.01$ ) and a high gastritis index ( $\tau = 0.33$ ;  $p < 0.01$ ).

**Table 3. Operated and nonoperated subjects assessed by multiple gastric biopsies to obtain a gastritis index**

	Vagotomy and drainage (n = 80)	Partial gastrectomy (n = 60)	Nonoperated (n = 78)
Age (years) <sup>a</sup>	57 (31-77)	64 (44-78)	60 (24-83)
Interval (years) <sup>a</sup>	15 (3-28)	20 (4-40)	
Gastritis index <sup>b</sup>	2.3 ± 0.08	2.6 ± 0.1	1.8 ± 0.08 <sup>c</sup>

<sup>a</sup>Median (range)

<sup>b</sup>Mean ± SE

<sup>c</sup>Operated versus nonoperated cases,  $p < 0.01$  (Mann-Whitney U test)

phic gastritis, as would be expected. This did not confer an increased risk on operated stomach patients, since high gastric pH occurred in nonoperated subjects and was again related to nitrite and chronic gastritis.

**Table 4. Gastritis index in operated subjects at different intervals after ulcer operation**

Interval (years)	Vagotomy and drainage		Partial gastrectomy	
	Number	Gastritis index (mean ± SE)	Number	Gastritis index (mean ± SE)
11-15	20	2.25 ± 0.1	12	2.4 ± 0.17
16-20	28	2.1 ± 0.12	9	2.2 ± 0.31
21-25	6	2.7 ± 0.53	8	3.0 ± 0.33 <sup>a</sup>
26 +	-	-	19	2.8 ± 0.18

<sup>a</sup>Gastritis index with operation interval < 20 years versus interval > 20 years,  $p < 0.05$  (Mann-Whitney U test)

#### Acknowledgement

This work was supported by grants from the Mersey Region Research Committee and the North West Cancer Research Fund, UK.

In this prospective endoscopic study of mucosal changes in operated stomach patients, there was no definite association between histological appearance and premalignancy. However, after ulcer surgery, there was increasing severity of chronic atrophic gastritis, which may be a dilute marker of increased malignant potential (Morson *et al.*, 1980). Despite this, only moderate degrees of gastritis were observed more than 20 years after gastrectomy, and unequivocal dysplasia was not seen. The nitrite level was correlated with pH and atro-

In summary, no increased RR was demonstrated in our case-control study for developing operated stomach cancer more than five years after surgery for benign ulcer. Moreover, changes observed in gastric mucosa after surgery probably corroborate this finding and cannot provide evidence that the operated stomach is a premalignant condition.

# RADIOIMMUNOASSAY USED TO DETECT DNA ALKYLATION ADDUCTS IN TISSUES FROM POPULATIONS AT HIGH RISK FOR OESOPHAGEAL AND STOMACH CANCER

C.P. Wild<sup>1</sup>, S.H. Lu<sup>2</sup> & R. Montesano<sup>1,3</sup>

<sup>1</sup>International Agency for Research on Cancer, 150 cours Albert-Thomas,  
69372 Lyon, France; and <sup>2</sup>Cancer Institute, Chinese Academy  
of Medical Sciences, Beijing, China

***N*-Nitrosamines are alkylating agents capable of forming various covalent adducts with DNA *in vivo*. Since formation of promutagenic DNA adducts, particularly *O*<sup>6</sup>-methyldeoxyguanosine (*O*<sup>6</sup>-medGuo), has been implicated as an initiating event in nitrosamine-induced carcinogenesis, we have used radioimmunoassay (RIA) to probe for such damage in surgical tissue samples from cancer patients in populations likely to be exposed environmentally to nitrosamines.**

## Populations selected for study

The criteria for selecting populations for study were that they have elevated rates of cancer of the oesophagus and/or stomach (Table 1) and that there be some evidence for exposure to environmental nitrosamines. Populations with low cancer incidence at these two sites were included for comparison.

Human exposure to nitrosamines may occur endogenously *via* in-vivo formation from precursor amines and nitrosating agents or directly by exposure to preformed compounds. In Lin-xian, China, the high incidence rates for oesophageal and stomach cancer contrast markedly with those in neighbouring provinces, and these differences have been suggested to result at least partially from variations in diet. Preformed nitrosamines, including *N*-nitrosodimethylamine, *N*-nitrosodiethylamine, *N*-nitroso-*N*-methylacetyl-*N*<sup>3</sup>-butylmethylamine and *N*-nitrosopyrrolidine (up to 1.5 ppb) have been identified in foods from villages in Lin-xian, as well as ppm levels of amines (Singer, G.M. *et al.*, 1986). In addition, higher urinary levels of *N*-nitrosamino acids and nitrate were found in subjects from Lin-xian when compared to subjects from Fan-xian, China, an area of low risk for oesophageal cancer (Lu *et al.*, 1986). In our preliminary studies (see below), we have found detectable levels of *O*<sup>6</sup>-medGuo in oesophageal and stomach mucosa obtained surgically from patients in Lin-xian which were higher than those in tissue specimens from areas of lower oesophageal cancer incidence in Europe.

These studies raised the questions of (i) whether there is a background level of alkylation in human tissues resulting from low-level exposure; (ii) whether that level is elevated specifically in target tissues of populations at high risk of developing a particular cancer and exposed to correspondingly higher levels of nitrosamines; and (iii) whether the same agents are implicated in elevated risks of oesophageal and stomach cancer in different areas of the world.

<sup>3</sup> To whom correspondence should be addressed

**Table 1. Oesophageal and stomach cancer incidences in populations under study<sup>a</sup>**

Population	Cancer incidence <sup>b</sup>			
	Oesophagus		Stomach	
	Male	Female	Male	Female
Lin-xian, China <sup>c</sup>	151.6	115.0	42.4	30.3
Normandy, France <sup>d</sup>	42.8	2.1	24.9	17.1
Singapore (Chinese)	18.9	4.1	43.7	17.6
Hong Kong	18.6	5.5	22.5	10.3
Japan	8-14	2-3	75-100	38-51
Cali, Colombia	3.1	1.7	46.3	27.3
Federal Republic of Germany	1-4	1-5	26-30	12-13
France (Bas-Rhin, Doubs)	13-17	0.7-0.8	17-20	6-8

<sup>a</sup>Data, except when indicated otherwise, are taken from Waterhouse *et al.* (1982).

<sup>b</sup>Age-standardized incidence rate per 100 000 per year except when stated

<sup>c</sup>Age-adjusted mortality rate per 100 000 per year from Office of Prevention and Treatment of Cancer (1978)

<sup>d</sup>From M. Gignoux, L. Chere & D. Pottier, personal communication

Levels of DNA alkylation in oesophageal and stomach tissue from the high-incidence areas of Lin-xian, Japan, Cali (Colombia) and Normandy (France) are therefore being compared with those in tissues obtained by surgery from cancer patients in Europe. The oesophageal samples from Singapore and Hong Kong are included for comparison, since these are Chinese populations at lower risk for oesophageal cancer.

### Immunoassay methodology

Formation of DNA adducts, particularly *O*<sup>6</sup>-alkyldeoxyguanosine and *O*-alkylpyrimidines, is likely to be an important factor in nitrosamine-induced carcinogenesis. If such promutagenic lesions are present in the DNA of tumour target organs, there would be further evidence for a causal relationship between exposure to alkylating agents, including nitrosamines, and certain types of human cancer. In addition, the endpoint being measured represents, at an individual level, the result of systemic distribution of carcinogens, the metabolic capacity of the tissues involved, the specificity and extent of DNA adducts formed and the efficiency of the DNA repair processes concerned.

RIA using monoclonal antibodies specific for *O*<sup>6</sup>-medGuo and *O*<sup>6</sup>-ethyldeoxyguanosine (*O*<sup>6</sup>-etdGuo) allow detection of 25 and 12.5 fmol adduct, respectively, per mg DNA (when 3 mg DNA are available). This methodology has been described recently (Umbenhauer *et al.*, 1985). Briefly, DNA is extracted from tissues using a phenol-chloroform/isoamyl alcohol method, enzymically hydrolysed and the modified and unmodified deoxynucleosides fractionated on an Aminex A7 cation-exchange resin column. Thus, pure samples of *O*<sup>6</sup>-medGuo and *O*<sup>6</sup>-etdGuo can be obtained for analysis by RIA.

Adducts at the  $O^6$  position of deoxyguanosine can rapidly be repaired by an alkyltransferase protein in mammalian tissues (for a recent review see Yarosh, 1985); therefore, any persistence of this adduct probably represents a fraction of the original extent of alkylation and an underestimate of total exposure. In order to quantify more stable adducts, we have developed a rabbit polyclonal antibody to  $O^4$ -methyldeoxythymidine ( $O^4$ -medT), a lesion formed initially in smaller quantities than  $O^6$ -medGuo but apparently less efficiently repaired and which may therefore accumulate to concentrations equal to or greater than those of  $O^6$ -medGuo under conditions of chronic exposure to methylating agents (Richardson *et al.*, 1985). The antibody was raised using  $O^4$ -methylthymidine ( $O^4$ -meT)-bovine serum albumin as an immunogen; its properties are presented in Table 2. In RIA, the limit of detection is 50 fmol  $O^4$ -meT per mg DNA. Using Aminex A7 chromatographic separation,  $O^4$ -meT co-chromatographs with deoxyadenosine. In order to obtain a pure sample prior to RIA, the  $O^4$ -meT-containing fractions are collected and re-chromatographed on a Lichosorb LC<sub>18</sub> reverse-phase column eluting isocratically with 15% methanol in water. The  $O^4$ -meT adduct, with a retention time of 29 min, is then well separated from deoxyadenosine (retention time, 12 min).

**Table 2. Specificity, sensitivity and affinity of polyclonal antibody to  $O^4$ -medT**

Inhibitor	Quantity required to give 50% inhibition of $^3\text{H}$ - $O^4$ -medT <sup>a</sup> -antibody binding in RIA (pmol)
$O^4$ -MedT	0.6
$O^4$ -Ethyldeoxythymidine	7.8
Deoxythymidine	$3.2 \times 10^5$
Deoxycytidine	$6.7 \times 10^5$
7-Methylguanosine <sup>b</sup>	$> 5 \times 10^5$
Deoxyguanosine <sup>c</sup> , deoxyadenosine <sup>d</sup>	$> 10^6$

<sup>a</sup>Specific activity, 16 Ci/mmol; prepared by Dr R. Saffhill, Paterson Laboratories, Manchester, UK

<sup>b</sup>24% inhibition with  $5 \times 10^5$  pmol

<sup>c</sup>27.6% inhibition with  $10^6$  pmol

<sup>d</sup>18.7% inhibition with  $10^6$  pmol

Affinity constant of antibody,  $0.8 \times 10^9$  l/mol

No  $O^6$ -etdGuo was detected in any of the samples, while, in contrast, eight samples of oesophageal mucosa contained levels between 59 and 161 fmol  $O^6$ -medGuo per mg DNA, and two stomach samples contained 89 and 62 fmol/mg DNA, respectively. A further 17 samples contained between 15 and 50 fmol/mg, and ten samples had levels below the limit of

This approach, of combined chromatography and RIA, allows measurements of  $O^6$ -medGuo,  $O^4$ -medT and  $O^6$ -etdGuo to be made in the same human tissue sample at fmol/mg concentrations of DNA, representing in the order of 100 molecules of adduct per average human diploid genome.

#### **$O^6$ -medGuo, $O^6$ -etdGuo and alkyltransferase in human tissues**

A total of 37 tissue specimens from Linxian (22 samples of oesophageal mucosa with no sign of tumour invasion, 11 samples of cardiac stomach mucosa and four of oesophageal tumours) of 2-12 g were initially analysed for  $O^6$ -medGuo and  $O^6$ -etdGuo as outlined above.

detection. In all tissues from Europe, less than 45 fmol/mg DNA were present, and seven of 14 were below the detection limit (Umbenhauer *et al.*, 1985). The levels of alkyltransferase protein in the tissues from Lin-xian were comparable to those reported for other gastrointestinal tissues from around the world (see Montesano *et al.*, 1985), suggesting that the persistence of *O*<sup>6</sup>-medGuo is unlikely to be the result of reduced cellular capacity to repair the adduct. However, this result does not exclude the possibility that the adduct is protected from repair in certain regions of DNA, which may be less accessible to the repair protein, or persists in a subpopulation of cells deficient in this protein. The persistence of a promutagenic lesion such as *O*<sup>6</sup>-medGuo in human tissues with an apparently large excess of repair protein raises some extremely interesting and important questions.



# DETERMINATION OF *N*-NITROSAMINES IN GASTRIC JUICE AND URINE AND A COMPARISON OF ENDOGENOUS FORMATION OF *N*-NITROSOPROLINE AND ITS INHIBITION IN SUBJECTS FROM HIGH- AND LOW-RISK AREAS FOR OESOPHAGEAL CANCER

S.H. Lu<sup>1,3</sup>, W.X. Yang<sup>2</sup>, L.P. Guo<sup>1</sup>, F.M. Li<sup>1</sup>, G.J. Wang<sup>1</sup>  
J.S. Zhang<sup>1</sup> & P.Z. Li<sup>1</sup>

<sup>1</sup>*Cancer Institute, Chinese Academy of Medical Sciences, Beijing;*  
*and* <sup>2</sup>*Cancer Institute, Chengchow, Henan Province, China*

Samples of gastric juice were collected from a high-risk area for oesophageal cancer in China and analysed for *N*-nitroso compounds. *N*-Nitrosodimethylamine (NDMA), *N*-nitrosodiethylamine (NDEA), *N*-nitrosomethylbenzylamine (NMBzA), *N*-nitrosopyrrolidine (NPYR) and *N*-nitrosopiperidine (NPIP) were identified in descending order of concentration. Several unknown compounds were also detected in the fasting gastric juice. A positive correlation was found between the amount of nitrosamines in gastric juice and the degree of severity of lesions of the oesophageal epithelium: the amounts of nitrosamines in gastric juice from subjects with a normal oesophageal epithelium were lower than those in subjects with marked dysplasia or carcinoma of the oesophagus. In addition, 1500 samples of 24-h urine were collected from various communes in six high-risk areas and two low-risk areas for this cancer in China and analysed for *N*-nitrosamino acids. Subjects in high-risk areas excreted higher levels than those in low-risk areas. Intake of L-proline resulted in marked increases in levels of urinary *N*-nitrosoproline (NPRO) in inhabitants from both high- and low-risk areas. Intake of moderate doses of vitamin C,  $\alpha$ -tocopherol and zinc by high-risk subjects reduced the urinary levels of *N*-nitrosamino acids to those found in undosed subjects in low-risk areas, suggesting a rational basis for prevention in high-risk areas.

In previous studies in Lin-xian county in China, a high-risk area for oesophageal cancer, we showed that (i) food from Lin-xian contained higher levels of *N*-nitrosamines than those from Fan-xian, a low-incidence area for oesophageal cancer (Lu *et al.*, 1978); (ii) pickled vegetables commonly consumed by Lin-xian residents contain several *N*-nitrosamines and their precursors, and there is a positive correlation between the consumption of pickled vegetables and incidence of oesophageal cancer (Lu, 1977, 1981, 1982; Wang *et al.*, 1983); (iii) there is a positive correlation between the amount of nitrite in well-water and in saliva of Lin-xian subjects and epithelial dysplasia and carcinoma of the oesophagus (Lu & Cheng, 1978; Wang *et al.*, 1979); and (iv) the urine of subjects from Lin-xian contained more *N*-nitrosamino acids and nitrates than that of Fan-xian subjects (Lu *et al.*, 1986). We have

<sup>3</sup> To whom correspondence should be addressed

now analysed *N*-nitrosamines in gastric juice of Lin-xian subjects.

In previous studies, we also demonstrated that the amounts of NPRO, *N*-nitrosothiazolidine 4-carboxylic acid (NTCA), *N*-nitrososarcosine (NSAR) and nitrate excreted in 24-h urine of undosed subjects in Lin-xian were significantly higher than those in Fan-xian. Ingestion of L-proline resulted in a marked increase in urinary NPRO levels in inhabitants from both areas, while intake of moderate doses of vitamin C by high-risk subjects effectively reduced their urinary levels of *N*-nitrosamino acids to those found in undosed subjects in the low-risk area (Lu *et al.*, 1986). In order to study geographical variation in exposure to *N*-nitroso compounds, the same parameters were measured in urine collected from inhabitants of Lin-xian, Lu Shan-xian and Jui-xian (Henan province), Yang Zhong-xian (Jiangsu province), Yuan Tian-xian (Sichuan province) and Nan Ao-xian (Guangdong province) — high-risk areas — and of Fan-xian and Yu-xian (Henan province) — low-risk areas.

### Materials and methods

**Collection of samples:** At the medical station of Lin-xian county, 353 gastric juice samples were taken through a rubber tube during endoscopy after an overnight fast. The samples were put into bottles containing 5 g ammonium sulfamate, packed immediately in dry ice and transported to Beijing, where they were deep frozen, pending analysis.

Samples of 24-h urine were collected in the spring of 1985 from healthy subjects living in various communes in the eight counties mentioned above. Urine specimens were collected according to four protocols: (a) from undosed subjects, in order to determine the background levels of *N*-nitrosamines; (b) from subjects who had ingested 100 mg L-proline three times a day 1 h after each meal; (c) from subjects who had ingested 100 mg L-proline together with 100 mg vitamin C or 100 mg  $\alpha$ -tocopherol three times a day 1 h after each meal; and (d) from subjects who had ingested 100 mg L-proline together with 5 mg zinc acetate three times a day 1 h after each meal. L-Proline, vitamin C and zinc were dissolved in 10 ml distilled water. Urine samples were collected from each subject immediately after a 24-h period in 2- to 3-l polypropylene bottles containing 10 g sodium hydroxide, mixed thoroughly, and the volume recorded. Two aliquots of 50 and 100 ml urine were then stored at  $-20^{\circ}\text{C}$  prior to analysis. *N*-Nitrosamines were stable under these storage conditions, and no appreciable artefact formation or degradation of the compounds was observed, even when samples to which nitrite had been added were stored at  $-20^{\circ}\text{C}$  for two years. Study subjects were asked to complete a questionnaire giving information on demography, food items eaten, and number of cigarettes or amount of Chinese pipe tobacco smoked during the 24 h of urine collection.

**Chemicals:** NPRO, NSAR and *N*-nitrosopipercolic acid (NPIC) were synthesized according to the method of Lijinsky *et al.* (1970); NTCA and *N*-nitroso(2-methylthiazolidine) 4-carboxylic acid (NMTCA) were prepared as described previously (Ohshima *et al.*, 1983, 1984a). Their purity was ascertained by thin-layer chromatography and gas chromatography coupled with either a thermal energy analyser, a flame ionization detector or a mass spectrometer. Diazomethane was prepared by the action of potassium hydroxide on *N*-nitroso-*N*-methyl-*p*-toluenesulfonamide (Merck, Darmstadt, Federal Republic of Germany) in diethylether. Other chemicals used were: L-proline (Sigma Chemical Co., St Louis, MO, USA), vitamin C, ammonium sulfamate, sulfanilic acid (Merck), ethyl acetate, methanol and dichloromethane (Beijing Chemical Industry).

**Analysis of *N*-nitrosamines:** For extraction of volatile nitrosamines, 5 ml gastric juice were diluted to 20 ml with distilled water, *N*-nitrosodipropylamine was added as internal standard, and the samples were extracted three times for 20 min with 40 ml dichloromethane in an agitator. The combined dichloromethane extracts were dried over anhydrous sodium sulfate, and the volume reduced to 2 ml in a Kuderna-Danish evaporator; 0.5 ml hexane was then added and the volume further reduced to about 0.5 ml.

For extraction of nonvolatile nitrosamines, NPRO and NSAR in urine were analysed initially according to a previously reported method, using ethyl acetate as the extraction solvent (Ohshima & Bartsch, 1981). For both gastric juice and urine samples, a 15-ml aliquot, to which NPIC had been added as internal standard, was extracted three times with 25 ml ethyl acetate in the presence of 5 g sodium chloride, 1.5 ml (gastric juice) or 3 ml (urine) 20% ammonium sulfamate solution in 3.6 N sulfuric acid. The residue was dissolved in 3 ml (gastric juice) or 2 ml (urine) diethylether and derivatized with *N*-nitroso-*N*-methyl-*p*-toluene sulfonamide (gastric juice) or with excess diazomethane (urine). The ethereal solution was then concentrated to 0.5 ml, and a 10- $\mu$ l aliquot was used to determine the methyl esters of NPRO and NSAR by gas chromatography-thermal energy analysis (GC-TEA).

**GC-TEA determination:** A Perkin-Elmer gas chromatograph was combined with a Thermal Energy Analyzer (TEA 502). A glass column (2 m  $\times$  3 mm i.d.) packed with 5% FFAP on Chromosorb W (80-100 mesh) was used at a temperature of 100-190°C; the carrier gas, argon, was passed at a flow rate of 25 ml/min; the temperature of the injection port of the GC was 220°C. The recoveries of NPRO and NSAR added to urine samples at 20  $\mu$ g/l were 85 and 82%, respectively.

## Results

***N*-Nitrosamines in gastric juice:** NDMA and NDEA were found in 95.2% and 94.3%, respectively, of the 353 samples of gastric juice from subjects in Lin-xian county (Table 1). These samples also contained several *N*-nitrosamines (tentatively identified as NMBzA, NPYR and NPIP) that can induce oesophageal cancer in animals (Druckrey *et al.*, 1967). NDMA occurred at the highest mean concentration, followed by NDEA, NMBzA, NPYR and NPIP. A high proportion of samples also contained NPRO and NSAR. A sex difference in concentration was seen, as gastric juice from male subjects contained 24.9  $\pm$  40.2 *N*-nitrosamines and that from females, 20.5  $\pm$  20.1 ng/ml.

**Table 1. *N*-Nitrosamines in gastric juice samples of Lin-xian subjects**

Nitrosamine	Positive findings		Concentration (ng/ml)
	No.	%	
NDMA	336/353	95.2	17.1 $\pm$ 53.3
NDEA	333/353	94.3	7.0 $\pm$ 59.1
NMBzA	40/353	11.3	4.8 $\pm$ 4.8
NPYR	61/353	17.3	2.5 $\pm$ 4.5
NPIP	38/353	10.8	1.3 $\pm$ 1.3
NPRO	27/74	32.4	0.6 $\pm$ 1.5
NSAR	35/74	47.3	2.3 $\pm$ 3.6
Unknown	33/74	44.6	4.9 $\pm$ 10.9

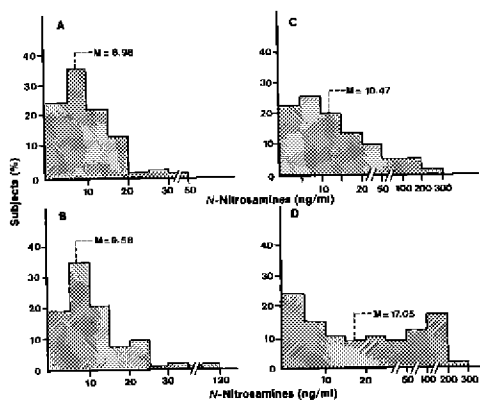
There was a clear positive trend between the amount of volatile *N*-nitrosamines in gastric juice and the severity of lesions of the oesophageal epithelium (Table 2), the amounts of NDMA, NDEA, NMBzA, NPYR and NPIP in gastric juice from subjects with normal oesophageal epithelium being lower than those from subjects with marked dysplasia and carcinoma of the oesophagus.

We also found a significant relation between pH and nitrosamine concentration in gastric

**Table 2. Correlation between severity of lesions of the oesophagus and *N*-nitrosamine contents (ng/ml) of gastric juice**

Group	No. of samples	NDMA	NDEA	NMBzA	NPYR	NPIP	Unknown compound	Total
Normal	50	3.72±3.04	6.83±5.60	0.11±0.40	0.10±0.29	0.02±0.07	0.33±0.99	11.10±8.27
Mild hyperplasia	45	6.21±16.32	6.68±5.48	0	0.11±0.25	0.05±0.18	1.12±3.98	14.11±22.57
Marked hyperplasia	155	16.04±44.70	5.86±9.00	0.42±2.66	0.81±3.11	0.11±0.58	2.64±10.79	26.01±60.11
Carcinoma	64	33.52±46.09	10.64±13.70	1.86±3.55	0.14±0.47	1.33±7.02	0.65±1.96	46.68±60.94

juice and oesophageal lesions. The average pH of gastric juice from patients with oesophageal cancer was  $2.09 \pm 1.69$  and their nitrosamine levels were higher, while the average pH of the gastric juice of normal subjects was  $4.36 \pm 2.30$ , and their nitrosamine levels were low. Furthermore, gastric juice from patients with conditions known to be associated with increased gastric acidity and reduced pH showed correspondingly higher nitrosamine levels in gastric juice. In patients with oesophageal cancer, the pH of gastric juice was 1-2 (Fig. 1).

**Fig. 1. Amounts of nitrosamines in gastric juice from Lin-xian subjects**

A, normal subject; B, subjects with mild oesophageal hyperplasia; C, subjects with marked oesophageal dysplasia; D, subjects with oesophageal carcinoma

ingested proline plus 15 mg zinc acetate, the levels of NPRO and total *N*-nitroso compounds were lower than those seen after ingestion of proline alone. Total *N*-nitrosamino acids and NPRO levels were also reduced below those with proline alone in subjects in Lu Shan-xian (high-risk) and Yu-xian (low-risk) who ingested proline plus  $\alpha$ -tocopherol.

*N-Nitrosamines in urine:* A relationship was seen between the risk for oesophageal cancer and the level of *N*-nitrosamines in 24-h urine samples from subjects living in the counties studied (Table 3). The levels of the individual *N*-nitroso compounds were also higher in subjects in various communes in five of the high-risk areas than in those in the low-risk area, Yu-xian (Table 4).

Intake of L-proline resulted in a marked increase in urinary NPRO excretion by subjects from all the counties studied but had no effect on the amounts of urinary NTCA or NSAR excreted. When the proline was taken together with 100 mg vitamin C, however, the urinary excretion of *N*-nitrosamines decreased to levels below that in undosed subjects in all high-risk areas, to a statistically significant extent ( $p < 0.05$ ). In subjects in two high-risk areas, Lin-xian and Nan Ao-xian, who

**Table 3. Correlation between mortality rate from oesophageal cancer and levels of *N*-nitrosamino acids in urine**

County	Mortality rate from oesophageal cancer/100 000 population	Level of sum of <i>N</i> -nitrosamino acids in urine ( $\mu\text{g}/\text{day}$ )
Lin-xian	151.6	36.8 $\pm$ 70.3
Hui-xian	138.0	33.9 $\pm$ 61.2
Yang Zhong-xian	100.0	18.5 $\pm$ 49.2
Nan Ao-xian	91.2	13.6 $\pm$ 18.1
Lu Shan-xian	76.2	21.3 $\pm$ 19.9
Yuan Tian-xian	71.2	13.7 $\pm$ 13.5
Fan-xian	35.1	7.9 $\pm$ 6.9
Yu-xian	26.7	6.3 $\pm$ 7.4

found in gastric juice from Lin-xian. These results indicate that inhabitants of Lin-xian are exposed to much higher amounts of the *N*-nitrosamines that induce oesophageal cancer in animals than are Europeans.

**Table 4. Urinary excretion of *N*-nitrosamines ( $\mu\text{g}/\text{day} \pm \text{SE}$ ) by subjects from five high-risk areas and a low-risk area for oesophageal cancer in China**

County	Treatment	No. of samples	NPRO	NSAR	NTCA	NMTCA	Unknown	Total
<i>High-incidence</i>								
Lin-xian	None	44	5.7 $\pm$ 8.2	0.1 $\pm$ 0.4 <sup>a</sup>	2.6 $\pm$ 4.9	ND	0.3 $\pm$ 1.3	8.7 $\pm$ 11.2
	Proline	47	10.2 $\pm$ 20.7	0.4 $\pm$ 0.9 <sup>a</sup>	3.9 $\pm$ 7.7	ND	0.4 $\pm$ 1.8	14.9 $\pm$ 24.1
	Proline + vitamin C	44	3.6 $\pm$ 5.9	0.1 $\pm$ 0.3 <sup>a</sup>	3.1 $\pm$ 8.0	ND	1.0 $\pm$ 3.2	8.2 $\pm$ 12.5
	Proline + zinc	44	4.7 $\pm$ 5.9	0.2 $\pm$ 0.7 <sup>a</sup>	5.4 $\pm$ 25.5	ND	0.7 $\pm$ 3.3	11.0 $\pm$ 27.8
Hui-xian	None	48	5.9 $\pm$ 9.2	2.6 $\pm$ 5.7	1.9 $\pm$ 6.1	0.1 $\pm$ 0.8	1.9 $\pm$ 3.5	12.4 $\pm$ 16.5
	Proline	47	12.2 $\pm$ 22.7	1.3 $\pm$ 2.1	1.0 $\pm$ 2.6	0.01 $\pm$ 0.08	1.6 $\pm$ 3.1	16.1 $\pm$ 23.3
	Proline + vitamin C	48	4.1 $\pm$ 4.7	1.1 $\pm$ 1.8	0.2 $\pm$ 0.6	0	3.6 $\pm$ 15.3	9.0 $\pm$ 15.7
Lu Shan-xian	None	54	4.2 $\pm$ 6.3	3.8 $\pm$ 8.9	4.5 $\pm$ 6.5	1.0 $\pm$ 4.8	7.8 $\pm$ 10.0	21.3 $\pm$ 19.9
	Proline	51	7.0 $\pm$ 11.8	3.4 $\pm$ 8.1	5.0 $\pm$ 12.1	0.8 $\pm$ 3.8	10.4 $\pm$ 13.2	26.6 $\pm$ 22.7
	Proline + vitamin C	52	2.8 $\pm$ 3.1	3.2 $\pm$ 7.1	3.3 $\pm$ 6.5	1.2 $\pm$ 5.8	4.5 $\pm$ 5.9	15.1 $\pm$ 14.3
	Proline + vitamin E	53	6.3 $\pm$ 8.7	1.5 $\pm$ 2.3	5.5 $\pm$ 11.1	1.4 $\pm$ 4.3	6.0 $\pm$ 8.6	18.4 $\pm$ 22.9
Yuan Tian-xian	None	57	6.6 $\pm$ 11.2	2.8 $\pm$ 3.1 <sup>a</sup>	2.6 $\pm$ 4.3	ND	1.7 $\pm$ 3.8	13.7 $\pm$ 13.7
	Proline	60	11.3 $\pm$ 24.9	2.7 $\pm$ 7.7 <sup>a</sup>	2.2 $\pm$ 3.9	ND	5.9 $\pm$ 22.9	21.9 $\pm$ 34.1
	Proline + vitamin C	59	3.5 $\pm$ 6.8	2.5 $\pm$ 7.4 <sup>a</sup>	2.8 $\pm$ 5.9	ND	3.2 $\pm$ 7.3	12.0 $\pm$ 14.3
Nan Ao-xian	None	50	4.4 $\pm$ 6.2	1.6 $\pm$ 8.5	6.7 $\pm$ 12.1	0.8 $\pm$ 3.8	0.8 $\pm$ 2.6	13.6 $\pm$ 18.1
	Proline	50	7.3 $\pm$ 8.3	0.1 $\pm$ 0.6	3.7 $\pm$ 4.1	0.4 $\pm$ 1.4	1.1 $\pm$ 3.5	12.5 $\pm$ 11.5
	Proline + vitamin C	47	4.6 $\pm$ 4.6	0.3 $\pm$ 0.9	4.3 $\pm$ 5.6	1.0 $\pm$ 5.2	0.2 $\pm$ 0.5	9.0 $\pm$ 8.0
	Proline + zinc	49	5.1 $\pm$ 5.1	0.4 $\pm$ 1.1	7.9 $\pm$ 7.8	0.6 $\pm$ 2.3	1.1 $\pm$ 3.6	16.2 $\pm$ 15.8
<i>Low incidence</i>								
Yu-xian	None	44	2.0 $\pm$ 2.6	1.5 $\pm$ 3.2	0.3 $\pm$ 1.6	1.3 $\pm$ 3.6	1.3 $\pm$ 4.3	6.3 $\pm$ 7.2
	Proline	49	3.4 $\pm$ 4.5	2.4 $\pm$ 4.6	1.0 $\pm$ 3.9	0.9 $\pm$ 3.9	0.9 $\pm$ 3.4	8.5 $\pm$ 8.3
	Proline + vitamin C	50	2.0 $\pm$ 3.2	2.7 $\pm$ 4.8	0.4 $\pm$ 2.2	0.8 $\pm$ 2.8	1.1 $\pm$ 2.4	6.7 $\pm$ 7.8
	Proline + vitamin E	45	3.1 $\pm$ 4.4	1.7 $\pm$ 2.8	0.03 $\pm$ 0.17	0.5 $\pm$ 1.7	0.9 $\pm$ 2.9	6.2 $\pm$ 7.2

<sup>a</sup> $\mu\text{g}/\text{h}$ 

ND, not determined

**Discussion**

Six *N*-nitroso compounds were identified in gastric juice from subjects in Lin-xian, at least four of which — NMBzA, NPYR, NPPI and NSAR — can induce oesophageal cancer in animals. Reed *et al.* (1981a,b) found only NDMA and NDEA in gastric juice collected in low-risk areas — Italy and the UK — at lower levels than those

The positive correlation seen between the amount of *N*-nitrosamines in gastric juice and the severity of lesions of the oesophageal epithelium in the subjects examined may indicate that they were more heavily exposed to the kinds and doses of *N*-nitrosamines that induce oesophageal lesions. This is the first demonstration in humans of a direct relationship between oesophageal cancer and a significantly raised concentration of *N*-nitroso compounds in gastric juice.

A main source of human exposure to *N*-nitroso compounds is synthesis *in vivo*; these compounds have been demonstrated in gastric juice of animals and humans (Mirvish, 1972; Li *et al.*, 1978; Reed *et al.*, 1981a,b). We consider that the *N*-nitroso compounds observed in gastric juice from Lin-xian were synthesized in stomach, since the levels were higher than those found in food in that area. The total concentration of *N*-nitroso compounds may in fact have been higher than that reported, since we made our measurements on fasting gastric juice, and some of the *N*-nitroso compounds formed in the stomach may have passed into the intestine or been absorbed by the gastric mucosa.

The results of the present study further demonstrate that inhabitants of high-risk areas for oesophageal cancer excrete higher levels of NPRO, NTCA and NSAR in their urine than do those of low-risk areas. The median level ( $\mu\text{g/day}$ ) of all *N*-nitrosamines found in the urine of high-risk subjects was about two to five times higher than that of low-risk subjects, suggesting that subjects in high-risk areas are exposed to higher levels of these compounds. Intake of vitamin C,  $\alpha$ -tocopherol or zinc in conjunction with proline reduced the urinary levels of NPRO and other *N*-nitrosamines in the urine of subjects from all areas, indicating that these factors effectively inhibit endogenous nitrosation. Vitamin C had the greatest effect.

The high levels of *N*-nitrosamines in the urine of subjects from high-risk areas for oesophageal cancer may be due either to a higher daily intake of nitrosating agents (nitrate, nitrite) or to higher levels of catalysts or lower levels of inhibitors in their diet. Nutritional surveys showed that inhabitants of Lin-xian consume few fresh vegetables and little fruit (Lu & Lin, 1982) and that the blood levels of zinc and various vitamins, such as vitamins C and A and riboflavin, were lower than in inhabitants of a low-risk area (Yang *et al.*, 1982). Fresh vegetables and fruits contain high concentrations of vitamin C and various other phenolic compounds, such as chlorogenic acid and tannins, all of which are generally inhibitors of *N*-nitrosation (Pignatelli *et al.*, 1984; Stich *et al.*, 1984b).

Our data suggest that inhabitants of the high-risk areas ingest higher levels of nitrosation agents and fewer nitrosation inhibitors, leading to more endogenous nitrosation. These findings offer a rational basis for prevention in these areas.

#### Acknowledgements

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# HIGH URINARY EXCRETION OF NITRATE AND N-NITROSOPROLINE IN OPISTHORCHIASIS SUBJECTS

S. Srianujata<sup>1,3</sup>, S. Tonbuth<sup>1</sup>, S. Bunyaratvej<sup>1</sup>, A. Valyasevi<sup>2</sup>,  
N. Promvanit<sup>2</sup> & W. Chaivatsagul<sup>2</sup>

<sup>1</sup>Research Center and Department of Pathology, Faculty of Medicine,  
Ramathibodi Hospital, and <sup>2</sup>Institute of Nutrition, Mahidol University,  
Bangkok 10400, Thailand

About 50 % of the population in some provinces of north-east Thailand are infested with liver fluke (*Opisthorchis viverrini*), and many develop cholangiocarcinoma subsequently. This study was designed to demonstrate possible endogenous formation of *N*-nitrosoproline (NPRO) in this population. Diet samples, saliva and urine were taken from subjects with and without liver fluke and analysed for nitrate and nitrite; urine was also analysed for NPRO. Nitrate and nitrite levels in saliva were higher in subjects with liver fluke than in those without; total nitrate and NPRO excretion was also higher in this group. Subjects with liver fluke may therefore be more heavily exposed to *N*-nitroso compounds than others, and may be at higher risk for cholangiocarcinoma.

This study is an extension of our previous study (Srianujata *et al.*, 1984) on possible endogenous formation of *N*-nitrosamines in subjects with liver fluke (*Opisthorchis viverrini*). Liver fluke has been found in approximately 50% of people in some areas of north-eastern Thailand, where cholangiocarcinoma is also prevalent (Bunyaratvej *et al.*, 1981). A synergistic activity has been found in Syrian golden hamsters given *N*-nitrosodimethylamine and liver fluke together (Thamavit *et al.*, 1978).

## Sample collection and analyses

Selected volunteers were divided into three groups: one from the north-east without liver fluke (20 subjects), another from the north-east with liver fluke (11 subjects), and the third consisting of normal subjects from Bangkok (12 subjects). All persons were allowed to consume normal meals, and duplicate portions of each meal, saliva before and after the meal, and 24-hour urine were collected from each subject. Nitrate and nitrite content were analysed in all samples using standard ISO methods (International Organization for Standardization, 1975a,b). NPRO was measured in urine according to the method developed by Ohshima (1983). Subjects were identified as infected by opisthorchis by total egg count in stools.

## Salivary nitrate and nitrite

The total food intake of Bangkok subjects was slightly higher than that of north-eastern groups, both with and without opisthorchiasis, while total nitrate and nitrite intake from food was slightly higher in the north-eastern groups (Table 1). Nitrate and nitrite levels in

<sup>3</sup> To whom correspondence should be addressed

saliva both before and after meals tended to be higher in subjects with opisthorchiasis, in agreement with our previous results (Srianujata *et al.*, 1984). Subjects with opisthorchiasis had higher salivary nitrate and nitrite levels than normal subjects living in the same area with the same dietary habits, even though the total average intakes of nitrate and nitrite from food were slightly lower. It is possible that endogenous formation of nitrate occurs in subjects whose livers are always infected by liver fluke; endogenous formation of nitrate has been shown to occur in various inflammatory conditions (Saul & Archer, 1984b; Wagner *et al.*, 1984b).

**Table 1. Age and total intake of food, nitrate and nitrite in three groups of subjects (mean  $\pm$  SE)**

	Bangkok	North-east	
		Normal	Opisthorchiasis
Age (years)	30.90 $\pm$ 2.00	35.65 $\pm$ 1.85	37.27 $\pm$ 2.78
Food intake (g/day)	1345.50 $\pm$ 118.10	1045.00 $\pm$ 71.30	1121.10 $\pm$ 82.80
Nitrate intake (mmol/day)	1.70 $\pm$ 0.33	3.06 $\pm$ 0.36	2.52 $\pm$ 0.48
Nitrite intake ( $\mu$ mol/day)	47.55 $\pm$ 11.85	89.50 $\pm$ 12.38	92.57 $\pm$ 31.26

from Bangkok. However, this may not be due to differences in dietary intake of nitrate, since it was shown by our previous study (Srianujata *et al.*, 1984) that the urinary concentration of nitrate was significantly higher in the northeastern group than in the Bangkok group, although the dietary intake of nitrate was comparable.

#### Urinary excretion of nitrate and NPRO

All subjects, both normal and with opisthorchiasis, were found to be free from urinary-tract infections, confirmed by bacterial culture of urine, as described elsewhere (Srianujata *et al.*, 1984). The average total volume of 24-h urine was practically the same in the three groups (Table 2). Nitrate levels in the urine of north-eastern subjects with opisthorchiasis were higher than those in normal subjects from that area and considerably higher than those

**Table 2. Urine volume, nitrate, nitrite and NPRO in the three groups of subjects (mean  $\pm$  SE)**

	Bangkok	North-east	
		Normal	Opisthorchiasis
Urine volume (ml/24 h)	1363 $\pm$ 271	1324 $\pm$ 184	1328 $\pm$ 332
Nitrite ( $\mu$ mol/24-h sample)	1.71 $\pm$ 0.38	2.73 $\pm$ 0.57	4.87 $\pm$ 1.52
Nitrate (mmol/24-h sample)	1.26 $\pm$ 0.21	1.10 $\pm$ 0.13	1.90 $\pm$ 0.48
NPRO (nmol/24-h sample)	33.57 $\pm$ 25.11	33.28 $\pm$ 5.53	85.85 $\pm$ 23.85
( $\mu$ g/24-h sample)	4.85 $\pm$ 1.15	4.80 $\pm$ 0.80	12.37 $\pm$ 3.44



Total 24-h excretion of NPRO was much higher in the north-eastern group with opisthorchiasis than in the normal north-eastern and Bangkok groups. It is conceivable that the group with opisthorchiasis has a greater risk of forming *N*-nitroso compounds endogenously when precursor amines are taken with foods. As was clearly demonstrated by Ohshima and Bartsch (1981), subjects with high intake of nitrate and proline excrete significantly more NPRO than usual.

This finding, in combination with the possible association between liver fluke and *N*-nitrosodimethylamine in causing cholangiocarcinoma (Thamavit *et al.*, 1978), may indicate that *N*-nitroso compounds play an important role in the development of cholangiocarcinoma in populations with liver fluke infection.

#### **Acknowledgements**

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## INTAKE OF CANTONESE-STYLE SALTED FISH AS A CAUSE OF NASOPHARYNGEAL CARCINOMA

M.C. Yu & B.E. Henderson

*Department of Preventive Medicine, University of Southern California School  
of Medicine, Los Angeles, CA, USA*

Ho (1971) of Hong Kong first proposed consumption of Cantonese-style salted fish, a traditional food among southern Chinese, as a possible risk factor for nasopharyngeal carcinoma (NPC) in this high-risk population. Four case-control studies have examined Ho's hypothesis; all results are supportive. The most convincing evidence of a causal association between intake of salted fish and NPC derives from a recent case-control study of young Hong Kong Chinese. It is estimated that over 90% of NPC cases under age 35 in Hong Kong are due to intake of this food during childhood. Preliminary experimental data on Cantonese-style salted fish indicate that *N*-nitroso compounds may be involved in the carcinogenicity of this human food.

### Epidemiological data

NPC is a rare malignancy in most parts of the world, with annual age-standardized incidence rates of less than 1 per 100 000 (Waterhouse *et al.*, 1982). A prominent exception is southern China, especially the central region of the province of Guangdong, where the annual age-standardized incidence in males is over 30 per 100 000 (Yu *et al.*, 1981). Southern Chinese immigrants to intermediate-risk areas such as south-east Asia, or low-risk areas such as the USA and Australia, continue to display a high risk for NPC (Worth & Valentine, 1967; Muir & Shanmugaratnam, 1967; Yu *et al.*, 1981). However, in the USA (Yu *et al.*, 1981) and Australia (Worth & Valentine, 1967), second-generation Chinese have an intermediate risk for NPC between those of first-generation Chinese and local whites. This situation is in contrast to that in Chinese in south-east Asia, where the native-born and the China-born have similar rates of NPC (Shanmugaratnam & Tye, 1970). Among south-east Asian Chinese, there is little assimilation to local cultures, whereas North American and Australian Chinese, especially those born in the host country, are likely to move away from the traditional life-style of southern China. The evidence, therefore, suggests that environmental factors inherent in the traditional culture of southern China are responsible for the high incidence of NPC among these people.

Fifteen years ago, Ho (1971) first proposed that intake of Cantonese-style salted fish, a favourite food among southern Chinese, might be a risk factor for NPC. The hypothesis is consistent with the descriptive features of this disease in Chinese. Cantonese-style salted fish is a popular food among southern Chinese, especially the people of Guangdong, but is not eaten by northern Chinese, who are at considerably lower risk for NPC. Among southern Chinese, NPC incidence is inversely associated with social class (Yu *et al.*, 1981), that is, lower social class individuals are more likely to develop NPC than higher social class individuals, and salted fish is traditionally one of the cheapest foods to supplement rice in

southern China. NPC is the most common cancer among young people of Hong Kong aged 15-34 (Ho, 1979), suggesting that exposure to the etiological agents occurs very early in life. Cantonese-style salted fish mixed with soft rice is a common food, sometimes the only food, fed to infants in the weaning and post-weaning period (Topley, 1973). In Hong Kong and Guangzhou, local fishermen, whose diet consists of large quantities of salted fish, have been shown to have a two-fold increased risk for NPC compared to the general population (Ho, 1978; Li, C.C. *et al.*, 1985).

Ho's hypothesis has been tested in four case-control studies conducted among southern Chinese. All are supportive of his hypothesis. In California (Henderson & Louie, 1978), 74 NPC patients and 109 hospital controls of Chinese ancestry were asked about current consumption of Cantonese-style salted fish. There was a statistically significant dose-response relationship with increasing frequency of consumption ( $p = 0.02$ ); individuals who ate the food more than once a week had a relative risk (RR) of 3.1 compared to nonconsumers.

In a case-control study of 150 NPC patients and 150 hospital controls in Hong Kong (Geser *et al.*, 1978), 108 mothers from NPC households and 103 from control households were interviewed regarding weaning habits. The only significant difference found in the type of food given to infants during and after weaning was Cantonese-style salted fish: 75% of mothers of NPC households fed their infants salted fish during weaning, compared to 53% of mothers of control households (RR = 2.6,  $p < 0.01$ ).

In Malaysia (Armstrong *et al.*, 1983), 100 Chinese NPC patients and 100 Chinese neighbourhood controls were interviewed regarding their frequency of consumption of Cantonese-style salted fish at the time of the interview, during adolescence, and during childhood. Cases and controls reported similar frequency of consumption around the time of the interview; however, significantly more cases than controls reported daily consumption of salted fish during adolescence and during childhood. The association was especially strong for consumption during childhood; the RR for daily relative to no consumption was 17.4 (95% confidence interval, 2.7-111.1). Consumption during adolescence was no longer a significant risk factor for NPC after controlling for consumption during childhood. It is estimated that over 60% of NPC cases in Malaysian Chinese are due to childhood consumption of this food.

The most convincing evidence linking Cantonese-style salted fish intake to the development of NPC in Chinese came from a recent case-control study of young NPC patients in Hong Kong (Yu *et al.*, 1986). The study was designed to investigate environmental exposures early in life, including diet, in relation to risk for NPC. A group of 250 incident cases of NPC under age 35 in Hong Kong Chinese and 250 controls were asked about their dietary habits three years previously and at age 10. In addition, 182 mothers of NPC patients and 155 mothers of controls were interviewed regarding the dietary habits of the study subjects at age 10, between ages one and two, and during weaning. Consumption of Cantonese-style salted fish at all time points was significantly associated with NPC (Table 1). The association was especially strong for childhood consumption; adult consumption was no longer significantly associated with NPC after childhood consumption had been accounted for. It is estimated that over 90% of NPC cases in Hong Kong Chinese under the age of 35 can be attributed to salted fish intake during childhood.

### Experimental data

Low levels (ppm or less) of volatile nitrosamines, including *N*-nitrosodimethylamine (NDMA), *N*-nitrosodiethylamine (NDEA), *N*-nitrosodi-*n*-propylamine (NDPA), *N*-nitro-

**Table 1. Frequency of salted fish consumption among NPC cases and controls in Hong Kong<sup>a</sup>**

Frequency	No. of cases	No. of controls	Relative risk	95% Confidence interval
Three years previously				
rarely	107	164	1.0	
monthly <sup>b</sup>	100	66	2.3	1.5-3.5
weekly <sup>c</sup>	37	19	3.2	1.7-6.1
daily	6	1	7.5	0.9-65.3
At age 10				
rarely	10	108	1.0	
monthly	125	101	15.0	6.0-37.2
weekly	113	39	37.7	14.1-100.4
Between ages 1-2				
never	19	83	1.0	
sometimes	65	34	6.1	3.0-12.5
often <sup>d</sup>	41	8	20.2	6.8-60.2
During weaning				
never	25	96	1.0	
ever	102	31	7.5	3.9-14.8

<sup>a</sup>Total may not add up to 250 case-control pairs or 127 case-mother/control-mother pairs due to missing values.

<sup>b</sup>Once a month to less than once a week

<sup>c</sup>Once a week to less than daily

<sup>d</sup>Considered by mothers to be a typical meal

sodi-*n*-butylamine (NDBA) and *N*-nitrosomorpholine (NMOR), have been detected in samples of Cantonese-style salted fish (Huang *et al.*, 1981). Most of these volatile nitrosamines, with the exception of NDMA, are potent inducers of nasal and paranasal cavity tumours in animals (Haas *et al.*, 1973; Pour *et al.*, 1973; Althoff *et al.*, 1974; Lijinsky & Taylor, 1978). However, the levels of preformed nitrosamines found in Cantonese-style salted fish are no higher than those detected in cured meats consumed in Europe, where the incidence of NPC is very low. Therefore, it is not clear whether any of the volatile nitrosamines detected in the fish is directly involved in the carcinogenic process.

More significantly, Huang *et al.* (1978b) conducted a small-scale experiment in which inbred Wistar albino rats fed Cantonese-style salted fish developed carcinomas of the nasal and paranasal regions. Twenty rats, aged one month, were fed steamed Cantonese-style salted fish (usual method of preparing this food in southern China) daily for six months and then given salted fish-head soup five days a week for the remainder of the experiment. All animals were sacrificed after two years or when moribund. Four of the 20 treated animals developed carcinomas in the nasal or paranasal regions, and none was observed among the six rats that served as controls.

We have initiated a similar experiment of a larger sample size: 219 inbred Wistar-Kyoto rats aged 21 days were randomly assigned within each sex to one of three experimental groups. Rats in group 1 are fed a powdered diet of one part salted fish and three parts rat chow during the first 18 months. Rats in group 2 are fed a powdered diet of one part salted fish and five parts rat chow during the same period. Rats in group 3 serve as controls; they are fed powdered rat chow only. As of week 99 of the experiment, three rats in group 1 have developed nasal cavity cancers. No other tumour of the respiratory tract has been observed in the other rats. We plan to follow these rats for three years.

## N-NITROSODIETHANOLAMINE EXCRETION IN METAL GRINDERS

B. Spiegelhalder<sup>1</sup>, J. Müller<sup>2</sup>, H. Drasche<sup>2</sup> & R. Preussmann<sup>1</sup>

<sup>1</sup>*Institute for Toxicology, German Cancer Research Center,  
Heidelberg; and* <sup>2</sup>*Institute of Occupational Medicine, Saarbrücken,  
Federal Republic of Germany*

Grinding fluids usually contain ethanolamines and nitrite as anticorrosive agents; these are known precursors of *N*-nitrosodiethanolamine (NDELA). In a preliminary study, it was demonstrated that workers' exposure to NDELA can be monitored by urine analysis. In order to estimate total daily exposure, 12 workers in a grinding shop were investigated by a three-step biological monitoring programme, giving the following results: (i) after exposure-free weekends, no NDELA was found in urine; (ii) urine collected during working shifts contained NDELA in up to  $\mu\text{g}/\text{kg}$  concentrations; (iii) total daily NDELA excretion in 24-h urines was up to 40  $\mu\text{g}$ ; (iv) the amount of excreted NDELA correlated with the amount of NDELA contamination in the grinding fluid; (v) NDELA seems to be accumulated in the body during the week; (vi) other workers in machine shops, like maintenance and transport workers, are also heavily exposed to NDELA.

Earlier studies showed that workers' exposure to NDELA can be monitored by urine analysis (Spiegelhalder *et al.*, 1984). In order to develop a simple urine assay for application in routine surveys, it was necessary to study the excretion characteristics of NDELA under controlled conditions.

### Method

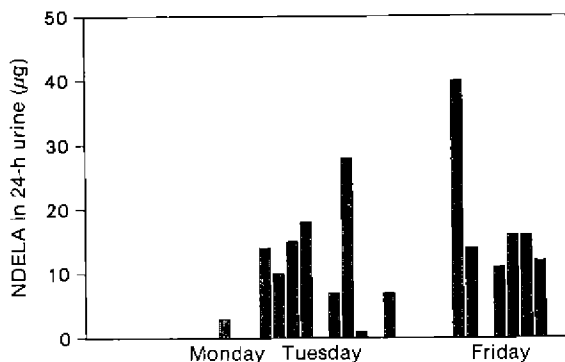
Samples of 0.5 g grinding fluid and 15 g urine were used for analysis. Nitrite was destroyed with sulfamic acid. NDELA was extracted using Kieselguhr extraction columns and ethyl formate containing 2% methanol; extracts were evaporated to dryness under a stream of nitrogen and the residue reacted with 0.3 ml *N*-methyl-*N*-trimethylsilylheptafluorobutyramide at 80°C for 2 h to obtain the NDELA-bis(trimethylsilyl)ether. Quantification was carried out by gas chromatography/chemiluminescence detection (TEA 502). The gas chromatograph conditions were: injector, 200°C; on-column injection; column, 0.635 cm o.d., 0.2 cm i.d.  $\times$  140 cm silanized borosilica glass filled with 6% OV275 on Volaspher A2 (Merck, FRG); oven, initial temperature 110°C, 5 min, temperature programme 10°/min, final temperature 220°C, 5 min.

Twelve workers in a grinding shop were investigated, comprising five operators, three transport workers and four workers with maintenance and regulating duties. They all had contact with machines and material contaminated with grinding fluid. Three sets of urines were collected: (i) random samples taken on Monday before the start of work, on Monday at the end of the shift and on Tuesday before the start of work; (ii) 24-h urines taken on Monday and Friday; and (iii) 8-h urines taken on Monday and Thursday during the work shift and 16-h urines taken subsequently. To avoid artefact formation during collection and storage, sampling bottles contained sodium hydroxide (15 g).

**Results**

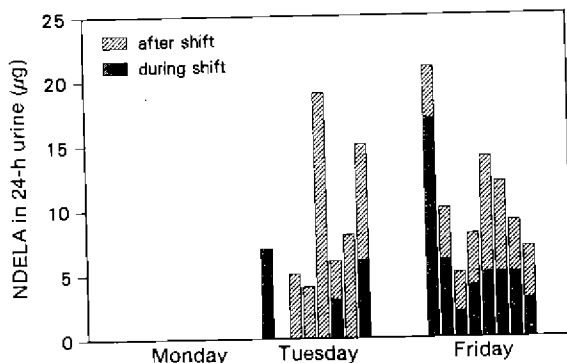
All cutting fluids used in the machine shop contained NDELA (5.5-25 ppm). In urine samples collected on Monday before the start of the first working shift, no NDELA was detected (only one urine contained 1 µg); however, NDELA exposure was already detected in some cases by the end of the first working shift following an exposure-free weekend. The number of NDELA-containing urines and the amount of excreted NDELA were higher at the end than at the beginning of a week. Quantitative estimations of NDELA in urines collected over defined time periods are shown in Figures 1 and 2. Table 1 gives mean urinary excretion of NDELA and the NDELA contamination of grinding fluids. Table 2 gives the mean NDELA excretion in different job descriptions. These results indicate increased NDELA excretion at the end of a working week. Earlier findings of a correlation between the extent of NDELA contamination in cutting fluid and the amount excreted in urine were thus confirmed.

**Fig. 1. Excretion of NDELA in 24-h urine of workers in a grinding shop**



Monday, excretion before start of work; Tuesday, urine collected during the first work shift on Monday and during the subsequent 16 h until Tuesday morning; Friday, urine collected from Thursday to Friday morning

**Fig. 2. Excretion of NDELA in urine of workers in a grinding shop**



During shift, collected over 8 h; after shift, collected over 16 h

**Table 1. NDELA excretion in urine of workers in a grinding shop in relation to NDELA content of grinding fluids**

NDELA in grinding fluid (mg/l)	NDELA in 24-h urine ( $\mu\text{g}$ )	
	Monday	Friday
25	$7.8 \pm 9.7$	$13.6 \pm 12.5$
11	$7.9 \pm 6.2$	$10.8 \pm 5.1$

**Table 2. NDELA excretion ( $\mu\text{g}$ ) in 24-h urine of workers in a grinding shop in relation to job description**

Time	Job		
	Operation	Transportation	Maintenance
Week's start	$5.1 \pm 9.7$	$8.3 \pm 9.7$	$10.9 \pm 4.9$
End of week	$15.3 \pm 10.4$	$8.7 \pm 7.6$	$9.4 \pm 8.3$

### Conclusions

NDELA is one nitrosamine for which biological monitoring can be used to detect occupational exposure. Results from quantitative excretion studies indicate accumulation of NDELA during a working week. During the weekend, however, NDELA is excreted completely. Representative results for exposure measurements by biological monitoring can be obtained from urines collected on Thursday or Friday at the end of a working shift. The use of nitrite-containing grinding fluids represents a greater risk for workers than was recognized earlier.

## N-MONONITROSOPIPERAZINE IN URINE AFTER OCCUPATIONAL EXPOSURE TO PIPERAZINE

T. Bellander<sup>1,3</sup>, B.-G. Osterdahl<sup>2</sup> & L. Hagmar<sup>1</sup>

<sup>1</sup>*Department of Occupational Medicine, Lund University Hospital,  
S-221 85 Lund; and* <sup>2</sup>*National Food Administration, Box 622,  
S-752 26 Uppsala, Sweden*

We have shown previously that intake of the drug piperazine can result in the endogenous formation of *N*-mononitrosopiperazine (NPZ; Bellander *et al.*, 1985). The present investigation deals with the possibility of endogenous formation of NPZ due to occupational exposure to piperazine in a chemical plant.

### Study groups and methods

Nine exposed individuals were monitored after a 12-h exposure. Two (Nos 1, 8) were also monitored after another 12 h (1b, 8b), and two were monitored again after a shorter exposure (2b, 3b). Controls were four individuals working in the plant, but judged not to be exposed to piperazine; two were monitored twice.

Breathing-zone samples were taken over the whole working day for determination of piperazine in air. Whole urine samples from exposed individuals were collected before, during and after exposure, for determinations of NPZ, piperazine, nitrite and nitrate. Saliva samples were obtained from both exposed and control workers before work, just before and 1 h after each meal and at the end of the working day, for analysis of nitrite and nitrate.

For determination of NPZ in urine, solid sodium hydroxide was added in advance to sample bottles (to give about 2 g/l) to prevent artefactual formation of nitrosamines. Four aliquots of 20 ml were drawn from each sample. After coding and less than 24-h storage at 4°C, two were sent to The National Food Administration, Uppsala, Sweden, and the remaining two to Pollock International Ltd, Reading, UK. Sample aliquots spiked with NPZ for recovery, or nitrite for artefact control, were included randomly in each shipment. No artefactual formation could be detected in samples with added nitrite.

At the National Food Administration, urine was analysed according to a previously described method (Bellander *et al.*, 1985): extraction into dichloromethane from a kieselguhr column, concentration and analysis by gas chromatography-thermal energy analysis (GC-TEA). External standards were applied. GC-TEA was also used at Pollock International, but after derivatization with trifluoroacetic anhydride (J.R.A. Pollock, personal communication). In this case, *N*-nitrosodi-*n*-propylamine was used as internal standard in the GC-TEA determination.

Sampling and analysis of piperazine in air, piperazine in urine, nitrite and nitrate in saliva and nitrite in urine were done as described previously (Bellander *et al.*, 1985). Nitrite was never detected in urine (< 0.5 mg/l).

<sup>3</sup>To whom correspondence should be addressed



### Nitrosamines in urine

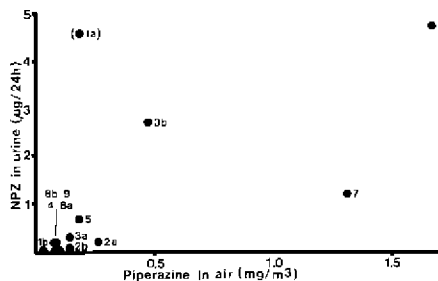
After five out of 13 exposures, NPZ was quantified in urine at total excretion levels of 0.7-4.7  $\mu\text{g}/\text{person}$  per 24 h; on five other occasions, traces of NPZ were detected in one or more urine samples; and, after the three remaining exposures, NPZ was not detected in any sample. The compound was not detected in controls, except for one out of 12 samples, where one out of four analyses showed the detection limit, 0.1 ng/ml. *N,N'*-Dinitrosopiperazine was not detected in either exposed or control workers.

After exposure No. 1a, analysis was performed by Pollock International only, without recovery control. This observation is therefore omitted from the following correlations between excretion of NPZ and other variables.

### Piperazine in air

The time-weighted average concentration of piperazine in the breathing zone over 12 h was  $< 0.03$ -1.7  $\text{mg}/\text{m}^3$  (median, 0.14;  $n = 13$ ) for exposed workers; the highest single 2-h value was 3.7  $\text{mg}/\text{m}^3$ . The level of piperazine in air showed a strong correlation (Spearman rank correlation coefficient,  $r_s = 0.86$ ;  $p < 0.001$ ;  $n = 12$ ) with that of NPZ in urine (Fig. 1).

Fig. 1. NPZ in urine and piperazine in air



Individual cumulated excretion of NPZ in urine after different exposure levels of piperazine in air (time-weighted average over 12 h); numbers: see text

### Nitrite and nitrate in saliva

The average concentration of nitrite in saliva was 7.1  $\text{mg}/\text{l}$  (SD, 3.5;  $n = 13$ ) for exposed and 6.8  $\text{mg}/\text{l}$  (SD, 2.8;  $n = 6$ ) for controls. The average concentration of nitrate in saliva was 17.0  $\text{mg}/\text{l}$  (SD, 10.9;  $n = 13$ ) for exposed and 15.5  $\text{mg}/\text{l}$  (SD, 8.0;  $n = 6$ ) for controls. Excretion of NPZ was correlated with both nitrite ( $r_s = 0.49$ ;  $p = 0.06$ ;  $n = 12$ ; Fig. 2) and nitrate in saliva ( $r_s = 0.49$ ;  $p = 0.06$ ;  $n = 12$ ).

Although five of the nine exposed workers were smokers (Nos 2, 3, 4, 6, 8), they did not excrete higher amounts of NPZ than the nonsmokers.

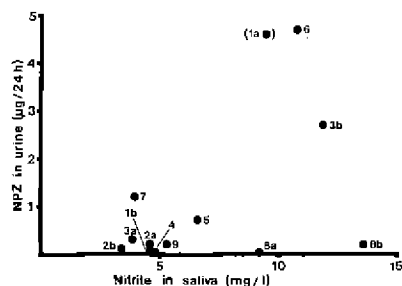
In controls, the concentration was always below the detection limit (0.05-0.3  $\text{mg}/\text{m}^3$ ), except for one sample, in which the detection limit (0.05  $\text{mg}/\text{m}^3$ ) was reached.

### Piperazine in urine

The level of excretion of piperazine in urine during the exposure and 12 h after was 70-4700  $\mu\text{g}/\text{person}$  (median, 430;  $n = 12$ ). On one occasion (No. 1b), the concentration in urine was below the detection limit, which, due to a large urine volume, corresponded to an excretion of 400  $\mu\text{g}$ . As for piperazine in air, the concentration of piperazine in urine showed a strong correlation ( $r_s = 0.74$ ;  $p = 0.003$ ;  $n = 12$ ) with that of NPZ in urine.

The controls did not excrete piperazine ( $< 0.20 \mu\text{g}/\text{ml}$ ).

Fig. 2. NPZ in urine and nitrite in saliva



Individual cumulated excretion of NPZ in urine at different nitrite levels in saliva; numbers: see text

lation there are indications of increased risks of malignant lymphoma/myelomatosis, lung cancer and bladder cancer (Hagmar *et al.*, 1986).

Discrepancies were seen in the results of the two laboratories, even after differences in recovery from spiked urines had been taken into account. For 11 samples, each with a joint sample mean  $> 1.0 \mu\text{g/l}$ , the individual analyses performed by Pollock International were 0.5 times higher (mean, SD 0.7) than the joint mean of the sample. This difference might be a result of metabolic conversion or conjugation of NPZ to a product which is recovered as NPZ in the respective analyses to varying extents.

Excretion of NPZ was also, as expected, correlated to levels of nitrite and nitrate in saliva.

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#### Discussion

To our knowledge, this is the first time that occupational exposure to an amine has been shown to cause endogenous formation of a potentially carcinogenic nitrosamine. Up to  $4.7 \mu\text{g}$  NPZ were excreted in urine over 24 h, and the excretion was strongly significantly correlated with individual exposure to piperazine.

Previous data suggested that only a minor part of the NPZ formed in the stomach is excreted in the urine (Bellander *et al.*, 1985); the fate of the remainder is not known: it may be metabolized or excreted *via* the faeces. The highest amount that could have been formed in this study was thus in the order of  $50 \mu\text{g/person}$ . It is noteworthy that in this popu-

## REFERENCES

## REFERENCES

- Abbott, P.J. & Saffhill, R. (1977) DNA synthesis with methylated poly(dA-dT) templates: possible role of  $O^6$ -methylthymine as a promutagenic base. *Nucleic Acids Res.*, *4*, 761-769
- Abdel-Tawab, G.A., Aboul-Azm, T., Edied, S.A. El-Toukhy, M.A., Abdel-Hamied, H.A., El-Kholy, Z.A. & El-Sharaky, A.S. (1986) The correlation between certain tryptophan metabolites and the *N*-nitrosamine content in the urine of bilharzial bladder cancer patients. *J. Urol.*, *135*, 826-830
- Acheson, E.D. & Doll, R. (1964) Dietary factors in carcinoma of the stomach: a study of 100 cases and 200 controls. *Gut*, *5*, 126-131
- Adams, J.D., Brunnemann, K.D. & Hoffmann, D. (1983) Rapid method for the analysis of tobacco-specific *N*-nitrosamines by GLC-TEA. *J. Chromatogr.*, *256*, 347-351
- Adams, J.D., LaVoie, E.J. & Hoffmann, D. (1985) On the pharmacokinetics of tobacco-specific *N*-nitrosamines in Fischer rats. *Carcinogenesis*, *6*, 509-511
- Airoldi, L., Bonfanti, M., Benfenati, E., Tavecchia, P. & Fanelli, R. (1983a) Identification of an acidic metabolite of *N*-nitrosodiethanolamine isolated from rat urine. *Biomed. Mass Spectr.*, *10*, 334-337
- Airoldi, L., Spagone, C., Bonfanti, M., Pantarotto, C. & Fanelli, R. (1983b) Rapid method for quantitative analysis of *N,N*-dibutyl nitrosamine, *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine and *N*-butyl-*N*-(3-carboxypropyl) nitrosamine in rat urine by gas chromatography-thermal energy analysis. *J. Chromatogr.*, *276*, 402-407
- Airoldi, L., Bonfanti, M., Fanelli, A., Bove, B., Benfenati, E. & Gariboldi, P. (1984) Identification of a nitrosamino aldehyde and a nitrosamino acid resulting from  $\beta$ -oxidation of *N*-nitrosodiethanolamine. *Chem.-biol. Interactions*, *51*, 103-113
- Albert, R.E., Sellakumar, A.R., Laskin, S., Kuschner, M., Nelson, N. & Snyder, C.A. (1982) Gaseous formaldehyde and hydrogen chloride induction of nasal cancer in rat. *J. natl Cancer Inst.*, *68*, 597-603
- Alexander, W.E., Ryan, A.J. & Wright, S.E. (1965) Metabolism of diphenylamine in the rat, rabbit and man. *Food Cosmet. Toxicol.*, *3*, 571-579
- Althoff, J., Mohr, U., Page, N. & Reznik, G.J. (1974) Carcinogenic effect of dibutyl nitrosamine in European hamsters (*Cricetus cricetus*). *J. natl Cancer Inst.*, *53*, 795-800
- Althoff, J., Grandjean, C., Russell, L. & Pow, P. (1977) Vinylethyl nitrosamine: a potent respiratory carcinogen in Syrian hamsters. *J. natl Cancer Inst.*, *58*, 439-442
- Althorpe, J., Goddard, D.A., Sissons, D.J. & Telling, G.M. (1970) The gas chromatographic determination of nitrosamines at the picogram level by conversion to their corresponding nitramines. *J. Chromatogr.*, *53*, 371-373
- Alwine, J.C., Kemp, D.J., Parker, B.A., Reiser, J., Renart, J., Stark, G.R. & Wahl, G.M. (1979) Detection of specific RNAs or specific fragments of DNA by fractionation in gels and transfer to diazobenzoyloxymethyl paper. *Meth. Enzymol.*, *68*, 220-242
- Amadori, D., Ravaioli, A., Gardini, A., Liverani, M., Zoli, W., Tonelli, B., Ridolfi, R. & Gentilini, P. (1980) *N*-Nitroso compound precursors and gastric cancer: preliminary data of a study on a group of farm workers. *Tumori*, *66*, 145-152
- Amano, K. & Yamada, K. (1964) A biological formation of formaldehyde in the muscle tissue of gadoid fish. *Bull. Jpn. Soc. Fish.*, *30*, 430-435

- Ames, B.N., McCann, J. & Yamasaki, E. (1975) Method for detecting carcinogens and mutagens with the *Salmonella*/mammalian microsome mutagenicity test. *Mutat. Res.*, *31*, 347-363
- Andersen, R.A. & Kemp, T.R. (1985) Accumulation of 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone in alkaloid genotypes of burley tobacco during postharvest processing: comparisons with *N*-nitrosornicotine and probable nitrosamine precursors. *Cancer Res.*, *45*, 5287-5293
- Andersen, R.A., Kasperbauer, M.J., Burton, H.R., Hamilton, J.L. & Yoder, E.E. (1982) Changes in chemical composition of homogenized leaf-cured and air-cured burley tobacco stored in controlled environments. *J. Agric. Food Chem.*, *30*, 663-668
- Andrews, A.W., Lijinsky, W. & Snyder, S.W. (1984) Mutagenicity of amine drugs and their products of nitrosation. *Mutat. Res.*, *135*, 105-108
- Anisimov, V.N. (1985) *Relevance of age to some aspects of carcinogenesis*. In: Likhachev, A., Anisimov, V. & Montesano, R., eds, *Age-related Factors in Carcinogenesis (IARC Scientific Publications No. 58)*, Lyon, International Agency for Research on Cancer, pp. 115-126
- Appel, K.E. & Graf, H. (1982) Metabolic nitrite formation from *N*-nitrosamines: evidence for a cytochrome P-450 dependent reaction. *Carcinogenesis*, *3*, 293-296
- Appel, K.E., Rickart, R., Schwarz, M. & Kunz, H.W. (1979a) Influence of drugs on activation and inactivation of hepatocarcinogenic nitrosamines. *Arch. Toxicol.*, *2* (Suppl.), 471-477
- Appel, K.E., Ruf, H.H., Mahr, B., Schwarz, M., Rickart, R. & Kunz, W. (1979b) Binding of nitrosamines to cytochrome P-450 of liver microsomes. *Chem.-biol. Interactions*, *28*, 17-33
- Appel, K.E., Schrenck, D., Schwarz, M., Mahr, B. & Kunz, W. (1980) Denitrosation of *N*-nitrosomorpholine by liver microsomes: possible role of cytochrome P-450. *Cancer Lett.*, *9*, 13-20
- Appel, K., Frank, N. & Wiessler, M. (1981) Metabolism of nitrosoacetoxymethylmethylamine in liver microsomes. *Biochem. Pharmacol.*, *30*, 2767-2772
- Appel, K.E., Rühl, C.S. & Hildebrandt, A.G. (1984a) *Metabolic inactivation of N-nitrosamines by cytochrome P-450 in vitro and in vivo*. In: O'Neill, I.K., von Borstel, R.C., Miller, C.T., Long, J. & Bartsch, H., eds, *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer (IARC Scientific Publications No. 57)*, Lyon, International Agency for Research on Cancer, pp. 443-451
- Appel, K.E., Rühl, C.S., Spiegelhalder, B. & Hildebrandt, A.G. (1984b) Denitrosation of diphenyl-nitrosamine *in vivo*. *Toxicol. Lett.*, *23*, 353-358
- Appel, K.E., Rühl, C.S. & Hildebrandt, A.G. (1985) Oxidative dealkylation and reductive denitrosation of nitrosomethylaniline *in vitro*. *Chem.-biol. Interactions*, *53*, 69-76
- Appel, K.E., Wiessler, M., Schoepke, M., Rühl, C.S. & Hildebrandt, A.G. (1986) Metabolic nitrite formation from *N*-nitrosamines: are there other pathways than reductive denitrosation by cytochrome P-450? *Carcinogenesis*, *7*, 659-663
- Archer, M.C. (1984) *Catalysis and inhibition of N-nitrosation reactions*. In: O'Neill, I.K., von Borstel, R.C., Miller, C.T., Long, J. & Bartsch, H., eds, *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer (IARC Scientific Publications No. 57)*, Lyon, International Agency for Research on Cancer, pp. 263-274
- Archer, M.C. & Eng, V.W.S. (1981) Quantitative determination of carcinogens and mutagens as alkylating agents following chemical activation. *Chem.-biol. Interactions*, *33*, 207-214
- Archer, M.C. & Labuc, G.E. (1985) *Nitrosamines*. In: Anders, M.W., ed., *Bioactivation of Foreign Compounds*, New York, Academic Press, pp. 403-431
- Archer, M.C., Tannenbaum, S.R., Fan, T.Y. & Weisman, M. (1975) Reaction of nitrite with ascorbate and its relation to nitrosamine formation. *J. natl Cancer Inst.*, *54*, 1203-1205
- Argus, M.F. & Hoch-Ligeti, C. (1961) Comparative study of the carcinogenic activity of nitrosamines. *J. natl Cancer Inst.*, *27*, 695-701
- Armijo, R., Gonzalez, A., Orellana, M., Coulson, A.H., Sayre, J.W. & Detels, R. (1981) Epidemiology of gastric cancer in Chile. II. Nitrate exposures and stomach cancer frequency. *Int. J. Epidemiol.*, *10*, 57-62

- Armitage, P. (1971) *Methods in Medical Research*, Oxford, Blackwell Press, pp. 203-204, 363-365
- Armstrong, R.W., Armstrong, M.J., Yu, M.C. & Henderson, B.E. (1983) Salted fish and inhalants as risk factors for nasopharyngeal carcinoma. *Cancer Res.*, **43**, 2967-2970
- Arshinov, V.Yu., Shulyakovskaya, T.S., Rykova, V.A. & Saprin, A.N. (1984) Role of butylhydroxytoluene in the metabolic inactivation of diethylnitrosamine. Investigation of the denitrosylating activity of the microsomes. *Dokl. Biochem.*, **274**, 23-25
- Arthur, D. (1972) Selenium content of Canadian foods. *Can. Inst. Food Sci. Technol. J.*, **5**, 165-169
- Auerbach, C., Moutschen-Dahmen, M. & Moutschen, J. (1977) Genetic and cytogenetical effects of formaldehyde and related compounds. *Mutat. Res.*, **39**, 317-367
- Autrup, H. & Stoner, G.D. (1982) Metabolism of *N*-nitrosamines by human and rat esophagus. *Cancer Res.*, **42**, 1307-1311
- Autrup, H., Harris, C.C. & Trump, B.F. (1978) Metabolism of acyclic and cyclic *N*-nitrosamines by cultured human colon. *Proc. Soc. exp. Biol. Med.*, **159**, 111-115
- Autrup, H., Jeffrey, A.M. & Harris, C.C. (1980) *Metabolism of benzo[a]pyrene in cultured human bronchus, trachea, colon, and esophagus*. In: BJORSETH, A. & DENNIS, A.J., eds, *Polynuclear Aromatic Hydrocarbons: Chemistry and Biological Effects*, Columbus, OH, Battelle Press, pp. 89-105
- Autrup, H., Grafstrom, R.C., Christensen, B. & Kieler, J. (1981) Metabolism of chemical carcinogens by cultured human and rat bladder epithelial cells. *Carcinogenesis*, **2**, 763-768
- Azoulay, E., Puig, J. & Martins Rosado de Sousa, M.L. (1969) Regulation of the synthesis of nitrate-reductase in *Escherichia coli* K<sub>12</sub> (Fr.). *Ann. Inst. Pasteur*, **117**, 474-485
- Ballet, F., Bouma, M.E., Wang, S.R., Amit, N., Marais, J. & Infante, R. (1984) Isolation, culture and characterization of adult human hepatocytes from surgical liver biopsies. *Hepatology*, **4**, 849-854
- Banfi, E., Tamaro, M. & Pani, B. (1974) Mechanism of the mutagenic activity of diazoacetylglycine derivatives. *Boll. Inst. Sieroter. Milan*, **53**, 632-635
- Banks, D.J. & Wiseman, P. (1968) Preparation of *N*-cyclohexyl-benzothiazole-2-sulphenamide by oxidative condensation. *J. appl. Chem.*, **18**, 262-265
- Bannasch, P., Moore, M.A., Klimek, F. & Zerban, H. (1982) Biological markers of preneoplastic foci and neoplastic nodules in rodent liver. *Toxicol. Pathol.*, **10**, 19-36
- Bannasch, P., Hacker, H.J., Klimek, F. & Mayer, D. (1984) *Hepatocellular glycogenosis and related pattern of enzymatic changes during hepatocarcinogenesis*. In: Weber, G., ed., *Advances in Enzyme Regulation*, Vol. 22, Oxford, Pergamon Press, pp. 97-121
- Baranyi-Furlong, B.L. & Goodman, J.I. (1984) Non-random interaction of AAF and its *N*-hydroxy metabolite with DNA of rat hepatocytic parenchymal and non-parenchymal cell nuclei following in vivo administration of carcinogen. *Chem.-biol. Interactions*, **48**, 15-28
- Barbacid, M. (1986) Oncogenes and human cancer. *Carcinogenesis*, **7**, 1037-1042
- Barnes, J.M. (1974) Nitrosamines. *Essays Toxicol.*, **5**, 1-15
- Bartholomew, B. & Hill, M.J. (1984) The pharmacology of dietary nitrate and the origin of urinary nitrate. *Food Chem. Toxicol.*, **22**, 789-795
- Bartsch, H. & Montesano, R. (1984) Relevance of nitrosamines to human cancer. *Carcinogenesis*, **5**, 1381-1393
- Bartsch, H., Ohshima, H., Muñoz, N., Crespi, M. & Lu, S.H. (1983a) *Measurement of endogenous nitrosation in humans: potential applications of a new method and initial results*. In: Harris, C.C. & Autrup, H.N., eds, *Human Carcinogenesis*, New York, Academic Press, pp. 833-855
- Bartsch, H., Terracini, B., Malaveille, C., Tomatis, L., Wahrendorf, J., Brun, G. & Dodet, B. (1983b) Quantitative comparison of carcinogenicity, mutagenicity and electrophilicity of 10 direct-acting alkylating agents and of the initial O<sup>6</sup>-7-alkylguanine ratio in DNA with carcinogenic potency in rodents. *Mutat. Res.*, **110**, 181-219

- Bartsch, H., Ohshima, H., Munoz, N., Crespi, M., Casale, V., Ramazzotti, V., Lambert, R., Minaire, Y., Forichon, J. & Walters, C.L. (1984) *In-vivo nitrosation, precancerous lesions and cancers of the gastrointestinal tract: on-going studies and preliminary results*. In: O'Neill, I.K., von Borstel, R.C., Miller, C.T., Long, J. & Bartsch, H., eds, *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer (IARC Scientific Publications No. 57)*, Lyon, International Agency for Research on Cancer, pp. 955-962
- Bate-Smith, E.C. (1972) Detection and determination of ellagitannins. *Phytochemistry*, *11*, 1153-1156
- Bavin, P.M.G., Darkin, D.W. & Viney, N.J. (1982) *Total nitroso compounds in gastric juice*. In: Bartsch, H., O'Neill, I.K., Castegnaro, M. & Okada, M., eds, *N-Nitroso Compounds: Occurrence and Biological Effects (IARC Scientific Publications No. 41)*, Lyon, International Agency for Research on Cancer, pp. 337-344
- Beach, R.S., Gershwin, M.E. & Hurley, L.S. (1982) Gestational zinc deprivation in mice: persistence of immuno-deficiency for three generations. *Science*, *218*, 469-471
- Beach, R.S., Gershwin, M.E. & Hurley, L.S. (1983) Persistent immunological consequences of gestation zinc deprivation. *Am. J. clin. Nutr.*, *38*, 579-590
- Becker, E.F., Jr, Zimmerman, B.K. & Geiduschek, E.P. (1964) Structure and function of cross-linked DNA. *J. mol. Biol.*, *8*, 377-391
- Becker, R.A. & Montesano, R. (1985) Repair of O<sup>6</sup>-methyldeoxythymidine residues in DNA by mammalian liver extracts. *Carcinogenesis*, *6*, 313-317
- Bedell, M.A., Lewis, J.G., Billings, K.C. & Swenberg, J.A. (1982) Cell specificity in hepatocarcinogenesis: O<sup>6</sup>-methylguanine preferentially accumulates in target cell DNA during continuous exposure to 1,2-dimethylhydrazine. *Cancer Res.*, *42*, 3079-3083
- Begert, A. (1981) *Expertise: Investigation of the Nitrosamine Burden in the Steyregg Area* (Prot. Nr 574/81) (Ger.), Bachmaning
- Beije, B., Janssen, D., Arrhenius, E. & Zetterqvist, M.-A. (1979) Isolated liver perfusion — a tool in mutagenicity and carcinogenicity testing for the evaluation of carcinogens. *Chem.-biol. Interactions*, *27*, 41-57
- Beije, B., Onfelt, A. & Olsson, U. (1984) Influence of dietary selenium on the mutagenic activity of perflusate and bile from rat liver, perfused with 1,1-dimethylhydrazine. *Mutat. Res.*, *130*, 121-126
- Beland, F.A., Dooley, K.L. & Casciano, D.A. (1979) Rapid isolation of carcinogen-bound DNA and RNA by hydroxyapatite chromatography. *J. Chromatogr.*, *174*, 177-186
- Belinsky, S.A., White, C.M., Boucheron, J.A., Richardson, F.C., Swenberg, J.A. & Anderson, M. (1986a) Accumulation and persistence of DNA adducts in respiratory tissue of rats following multiple administration of the tobacco-specific carcinogen 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone. *Cancer Res.*, *46*, 1280-1284
- Belinsky, S.A., White, C.M., Devereux, T.R., Swenberg, J.A. & Anderson, M.W. (1986b) Low dose accumulation of O<sup>6</sup>-methylguanine in lung following multiple dose administrations of the tobacco-specific carcinogen 4-(N-methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). *Proc. Am. Assoc. Cancer Res.*, *27*, 103
- Belinsky, S.A., White, C.M., Devereux, T.R., Swenberg, J.A. & Anderson, M.W. (1986c) Cell selective alkylation of DNA in lung following low dose exposure to a tobacco specific carcinogen. *Cancer Res.* (in press)
- Belinsky, S.A., Walker, V.E., Maronpot, R.R., White, C.M., Deal, F.H., Swenberg, J.A. & Anderson, M.W. (1986d) Molecular dosimetry for DNA adduct formation and cell toxicity in nasal mucosa following exposure to the tobacco specific carcinogen 4-(N-methyl-N-nitrosamino)-1-(3-pyridyl)-1-butanone. *Carcinogenesis* (submitted)
- Bellander, T., Österdahl, B.-G. & Hagmar, L. (1985) Formation of N-mononitrosopiperazine in the stomach and its excretion in the urine after oral intake of piperazine. *Toxicol. appl. Pharmacol.*, *80*, 193-198

- Berger, H., Habs, M. & Schmähl, D. (1983) Noncarcinogenic chemotherapy with a combination of vincristine, methotrexate and 5-fluorouracil (VMF) in rats. *Int. J. Cancer*, *32*, 231-236
- Berkowitz, E.M.L. & Silk, H. (1981) Methylation of chromosomal DNA by two alkylating agents differing in carcinogenic potential. *Cancer Lett.*, *12*, 311-321
- Bernfeld, P., Homburger, F., Soto, E. & Pai, K.J. (1979) Cigarette smoke inhalation studies in inbred Syrian golden hamsters. *J. natl Cancer Inst.*, *63*, 675-689
- Bertram, B., Schuhmacher, J., Frei, E., Frank, N. & Wiessler, M. (1982) Effects of disulfiram on mixed function oxidase systems and trace element concentration in the liver of rats. *Biochem. Pharmacol.*, *31*, 3613-3619
- Bhattacharjee, S.B. & Pal, B.F. (1982) Tetracycline induced mutation in cultured Chinese hamster cells. *Mutat. Res.*, *101*, 329-338
- Bhide, S.V., Shivapurkar, N.M., Gothoskar, S.V. & Ranadive, K.J. (1979) Carcinogenicity of betel quid ingredients: feeding of mice with aqueous extract and the polyphenol fraction of betel nut. *Br. J. Cancer*, *40*, 922-926
- Bhide, S.V., Murdia, U.S. & Nair, J. (1984) Polycyclic aromatic hydrocarbon profiles of pyrolysed tobacco products commonly used in India. *Cancer Lett.*, *24*, 80-94
- Bhide, S.V., Nair, U.J., Nair, J., Spiegelhalder, B. & Preussmann, R. (1986) *N*-Nitrosamines in the saliva of tobacco chewers or masheri users. *Food chem. Toxicol.*, *24*, 293-297
- Birnbaum, G.I., Sadana, K.L., Blonski, W.J.P. & Hruska, F.E. (1986) Carcinogenic alkylation of nucleic acid bases. Structure and conformation of *O*<sup>6</sup>-ethyl-2'-deoxythymidine in the solid state and in solution. *J. Am. chem. Soc.*, *108*, 1671-1675
- Bjelke, E. (1979) Epidemiologic studies of cancer of the stomach, colon and rectum; with special emphasis on the role of diet. *Scand. J. Gastroenterol.*, *9* (Suppl.), 1-235
- Bjelke, E. (1980) Epidemiological problems in the genetics of stomach cancer. *Nordic Council Arct. med. Res. Rep.*, *26*, 98-102
- Blackwell, B. & Mabbitt, L.A. (1965) Tyramine in cheese related to hypertensive crises after monoamine-oxidase inhibition. *Lancet*, *i*, 938-940
- Blot, W.J. & Li, J.-Y. (1985) Some considerations in the design of a nutrition intervention trial in Lin-xian China. *Natl Cancer Inst. Monog.*, *69*, 29-34
- Blouin, A. & Cormier, Y. (1986) Endotracheal intubation in guinea pigs by direct laryngoscopy. *Lab. Anim. Sci.* (in press)
- Böckler, R., Meyer, H. & Schlag, P. (1983) An experimental study on bacterial colonization, nitrite and nitrosamine production in the operated stomach. *J. Cancer Res. clin. Oncol.*, *105*, 62-66
- Bodell, W.J. (1977) Non-uniform distribution of DNA repair in chromatin after treatment with methylmethane sulphonate. *Nucleic Acids Res.*, *4*, 2619-2628
- Bogden, J.M., Eastman, A. & Bresnick, E. (1981) A system in mouse liver for the repair of *O*<sup>6</sup>-methylguanine lesions in methylated DNA. *Nucleic Acid Res.*, *9*, 3089-3103
- Bögemann, M., Petersen, S., Schultz, O.-E. & Söll, H. (1955) *Thiocarbamide acids and their derivatives* (Ger.). In: Müller, E., ed., *Houben-Weyl, Methoden der organischen Chemie*, Vol. IX, Stuttgart, G. Thieme, pp. 823-827
- Bognar, R. & Puskas, M.M. (1973) *N*-Glycosides. XVII. Preparation and structure of *N*-nitroso *N*-aryl glycosylamines. *Acta chem. acad. sci. hung.*, *76*, 399-407
- Boido, V., Bennicelli, C., Zanacchi, P. & De Flora, S. (1980) Formation of mutagenic derivatives from nitrite and two primary amines. *Toxicol. Lett.*, *6*, 379-383
- Bonney, G.E., Elston, R.C., Correa, P., Haenszel, W., Zavala, D.E., Zarama, G., Collazos, T. & Cuello, C. (1986) Genetic etiology of gastric carcinoma. I. Chronic atrophic gastritis. *Genetic Epidemiol.*, *3*, 213-224
- Borm, P.J.A., Koster, A.S., Frankhuijzen-Sierevogel, A.C. & Noordhoek, J. (1983) Comparison of two cell isolation procedures to study in vitro intestinal wall biotransformation in control and 3-methylcholanthrene pretreated rats. *Cell Biochem. Funct.*, *1*, 161-167



- Boutwell, R.K. (1974) The function and mechanism of promoters of carcinogenesis. *CRC crit. Rev. Toxicol.*, 2, 419-443
- Boyd, M.R. (1977) Evidence for the Clara cells as a site of cytochrome P-450-dependent mixed-function oxidase activity in the lungs. *Nature*, 269, 713-715
- Boyland, E., Carter, R.L., Gorrod, J.W. & Roe, F.J.C. (1968) Carcinogenic properties of certain rubber additives. *Eur. J. Cancer*, 4, 233-239
- Bradley, M.O. & Sina, J.F. (1984) *Methods for detecting carcinogens and mutagens with the alkaline elution/rat hepatocyte assay*. In: Kilbey, F.J., Legator, M., Nichols, W. & Ramel, C., eds, *Handbook of Mutagenicity Test Procedures*, Amsterdam, Elsevier, p. 71
- Bradley, M.O., Dysart, G., Fitzsimmons, K., Harbach, P., Lewin, J. & Wolf, G. (1982) Measurement by filter elution of DNA single- and double-strand breaks in rat hepatocytes: effects of nitrosamines and gamma-irradiation. *Cancer Res.*, 42, 2592-2597
- Brambilla, G., Cavanna, M., Parodi, S. & Caraceni, C.E. (1970) Carcinogenic activity in neonatal Swiss mice of *N*-diazooacetyl derivatives of glycine (Ital.). *Boll. Sci. It. Biol. Sper.*, 46, 127-131
- Brambilla, G., Cavanna, M., Parodi, S. & Baldini, L. (1972) Induction of tumours in newborn and adult Swiss mice by *N*-diazooacetyl glycine amide. *Eur. J. Cancer*, 8, 127-129
- Bratton, A.C. & Marshall, E.K. (1939) A new coupling component for sulfanilamide determination. *J. biol. Chem.*, 128, 537-550
- Braven, J., Bonker, G.J., Fenner, M.L. & Tonge, B.L. (1967) The mechanism of carcinogenesis by tobacco smoke: some experimental observations and hypothesis. *Br. J. Cancer*, 21, 623-633
- Brennan, R.G., Pyzalska, D., Blonski, W.J.P., Hruska, F.E. & Sundaralingam, M. (1986) Crystal structure of the promutagen *O*<sup>6</sup>-methylthymidine: importance of the anti conformation of the *O*(4)methoxy group and possible mispairing of *O*<sup>6</sup>-methylthymidine with guanine. *Biochemistry*, 25, 1181-1185
- Brent, T.P. (1986) Inactivation of purified human *O*<sup>6</sup>-alkylguanine-DNA alkyltransferase by alkylating agents or alkylated DNA. *Cancer Res.*, 46, 2320-2323
- Brent, T.P., Houghton, P.J. & Houghton, J.A. (1985) *O*<sup>6</sup>-Alkylguanine-DNA-alkyltransferase activity correlates with the therapeutic response of human rhabdomyosarcoma xenografts to 1-(2-chloroethyl)-3-(*trans*-4-methylcyclohexyl)-1-nitrosourea. *Proc. natl Acad. Sci. USA*, 82, 2985-2989
- Breslow, N.E. & Day, N.E. (1980) *Statistical Methods in Cancer Research*, Vol. 1, *The Analysis of Case-Control Studies (IARC Scientific Publications No. 32)*, Lyon, International Agency for Research on Cancer
- Brinton, L.A., Blot, W.J., Becker, J.A., Winn, D.M., Browder, J.P., Farmer, J.C. & Fraumeni, J.F., Jr (1984) A case-control study of cancers of the nasal cavity and paranasal sinuses. *Am. J. Epidemiol.*, 119, 896-906
- Brittebo, E.B. & Tjälve, H. (1981) Formation of tissue-bound *N*'-nitrosornicotine metabolites by the target tissues of Sprague-Dawley and Fischer rats. *Carcinogenesis*, 2, 959-963
- Brittebo, E.B., Castonguay, A., Furuya, K. & Hecht, S.S. (1983) Metabolism of tobacco-specific nitrosamines by cultured rat nasal mucosa. *Cancer Res.*, 43, 4343-4348
- van Broekhoven, L.W., Davies, J.A.R. & Geurink, J.H. (1984) *N*-Nitrosoproline excretion in urine, faeces and milk from cows in relation to feed composition. In: O'Neill, I.K., von Borstel, R.C., Miller, C.T., Long, J. & Bartsch, H., eds, *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer (IARC Scientific Publications No. 57)*, Lyon, International Agency for Research on Cancer, pp. 205-212
- Brooks, J.B., Cherry, W.B., Thacker, L. & Alley, C.C. (1972) Analysis by gas chromatography of amines and nitrosamines produced *in vivo* and *in vitro* by *Proteus mirabilis*. *J. infect. Dis.*, 126, 143-153

- Brunnemann, K.D., Scott, J.C., Haley, N.J. & Hoffmann, D. (1984) *Endogenous formation of N-nitrosoproline upon cigarette smoke inhalation*. In: O'Neill, I.K., von Borstel, R.C., Miller, C.T., Long, J. & Bartsch, H., eds, *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer (IARC Scientific Publications No. 57)*, Lyon, International Agency for Research on Cancer, pp. 819-828
- Brunnemann, K.D., Genoble, L. & Hoffmann, D. (1986) Identification and analysis of a new tobacco-specific N-nitrosamine 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanol in snuff tobacco. *Carcinogenesis* (submitted)
- Buege, J.A. & Aust, S.D. (1978) Microsomal lipid peroxidation. *Meth. Enzymol.*, 52, 302-310
- Bulay, O. & Mirvish, S.S. (1979) Carcinogenesis in rat esophagus by intraperitoneal injection of different doses of methyl-n-amyl nitrosamine. *Cancer Res.*, 39, 3644-3646
- Bunyaratvej, S., Meenakanit, V., Tantachamrun, T., Sriawat, P., Susilaworn, P. & Chongchitnan, N. (1981) National survey of major diseases in Thailand. Analysis of 3,305 biopsies as to year-end 1978. *J. med. Assoc. Thailand*, 64, 432-439
- Bursch, W., Lauer, B., Timmermann-Trosiener, I., Barthel, G., Schuppler, J. & Schulte-Hermann, R. (1984) Controlled death (apoptosis) of normal and putative preneoplastic cells in rat liver following withdrawal of tumor promoters. *Carcinogenesis*, 5, 453-458
- Busby, W.F., Jr, Shuker, D.E.G., Charnley, G., Newberne, P.M., Tannenbaum, S.R. & Wogan, G.N. (1985) Carcinogenicity of the nitrosated bile acid conjugates, N-nitrosoglycocholic acid and N-nitrosotaurocholic acid. *Cancer Res.*, 45, 1367-1371
- Bustad, L.K. (1966) Pigs in the laboratory. *Sci. Am.*, 214, 94-100
- Byers, T., Vena, J., Mettlin, C., Swanson, M. & Graham, S. (1984) Dietary vitamin A and lung cancer risk: an analysis by histologic subtypes. *Am. J. Epidemiol.*, 120, 769-776
- Byler, D.M., Gosser, D.K. & Susi, H. (1983) Spectroscopic estimation of the extent of S-nitrosothiol formation by nitrite action on sulfhydryl groups. *J. Agric. Food chem.*, 31, 523-527
- Calmels, S., Ohshima, H., Vincent, P., Gounot, A.-M. & Bartsch, H. (1985) Screening of microorganisms for nitrosation catalysis at pH 7 and kinetic studies on nitrosamine formation from secondary amines by *E. coli* strains. *Carcinogenesis*, 6, 911-915
- Cardy, R.H., Lijinsky, W. & Hildebrandt, P.K. (1979) Neoplastic and non-neoplastic urinary bladder lesions induced in Fischer 344 rats and B6C3F1 hybrid mice by N-nitrosodiphenylamine. *Ecotoxicol. environ. Saf.*, 3, 29-35
- Castegnaro, M., Massey, R.C. & Walters, C.L. (1986) The collaborative evaluation of a procedure for the determination of N-nitroso compounds as a group. *Food Addit. Contam.* (in press)
- Castonguay, A., Stoner, G.D., Schut, H.A.J. & Hecht, S.S. (1983a) Metabolism of tobacco-specific N-nitrosamines by cultured human tissues. *Proc. natl Acad. Sci. USA*, 80, 6694-6697
- Castonguay, A., Tjälve, H. & Hecht, S.S. (1983b) Tissue distribution of the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, and its metabolites in F344 rats. *Cancer Res.*, 43, 630-638
- Castonguay, A., Tharp, R. & Hecht, S.S. (1984) *Kinetics of DNA methylation by the tobacco specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in the F344 rat*. In: O'Neill, I.K., von Borstel, R.C., Miller, C., Long, J. & Bartsch, H., eds, *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer (IARC Scientific Publications No. 57)*, Lyon, International Agency for Research on Cancer, pp. 805-810
- Caygill, C.P.J., Hill, M., Craven, J., Hall, R. & Miller, C. (1984) *Relevance of gastric achlorhydria to human carcinogenesis*. In: O'Neill, I.K., von Borstel, R.C., Miller, C.T., Long, J. & Bartsch, H., eds, *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer (IARC Scientific Publications No. 57)*, Lyon, International Agency for Research on Cancer, pp. 895-900
- Caygill, C.P.J., Hill, M.J., Kirkham, J.S. & Northfield, T.C. (1986) Mortality from gastric cancer following gastric surgery for peptic ulcer. *Lancet*, i, 929-931

- Cerutti, P.A. (1985) Prooxidant states and tumor production. *Science*, 227, 375-381
- Challis, B.C. & Challis, J.A. (1982) *N-Nitrosamines and N-nitrosimines*. In: Patai, S., ed., *The Chemistry of Amino, Nitroso and Nitro Compounds and their Derivatives*, New York, Wiley, pp. 1151-1223
- Challis, B.C. & Kyrtopoulos, S.A. (1979) Nitrosation of amines by the two-phase interaction of amines in solution with gaseous oxides of nitrogen. *J. Chem. Soc. Perkin Trans. I*, 299-304
- Challis, B.C. & Outram, J.R. (1982) Formation of *N*-nitrosamines in solution from dissolved nitric oxide in the presence of HI or metal iodides. *J. chem. Soc. Perkin Trans. II*, 693-699
- Challis, B.C., Outram, J.R. & Shuker, D.E.G. (1980) *New pathways for the rapid formation of N-nitrosamines under neutral and alkaline conditions*. In: Walker, E.A., Griciūtė, L., Castegnaro, M. & Börzsönyi, M., eds, *N-Nitroso Compounds: Analysis, Formation and Occurrence (IARC Scientific Publications No. 31)*, Lyon, International Agency for Research on Cancer, pp. 43-56
- Challis, B.C., Lomas, S.J., Rzepa, H.S., Bavin, P.M.G., Darkin, D.W., Viney, N.J. & Moore, P.J. (1982) *A kinetic model for the formation of gastric N-nitroso compounds*. In: Magee, P.N., ed., *Nitrosamines and Human Cancer (Banbury Report 12)*, Cold Spring Harbor, NY, CSH Press, pp. 243-256
- Challis, B.C., Milligan, J.R. & Mitchell, R.C. (1984) Synthesis and stability of *N*-nitrosodipeptides. *J. chem. Soc. chem. Commun.*, 1050-1051
- Chang, R.L., Huang, M.-T., Wood, A.W., Wong, C.-Q., Newmark, H.L., Yagi, H., Sawyer, J.M., Jerina, D.M. & Conney, A.H. (1985) Effect of ellagic acid and hydroxylated flavonoids on the tumorigenicity of benzo[*a*]pyrene and (±)-7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene on mouse skin and in the newborn mouse. *Carcinogenesis*, 6, 1127-1133
- Chang, S.K., Harrington, G.W., Rothstein, M., Shergalis, W.A., Swern, D. & Vohra, S.K. (1979) Accelerating effect of ascorbic acid on *N*-nitrosamine formation and nitrosation by oxyhyponitrite. *Cancer Res.*, 39, 3871-3874
- Chang, T.M.S., MacIntosh, F.C. & Mason, S.G. (1966) Semi-permeable aqueous microcapsules. I. Preparation and properties. *Can. J. Physiol. Pharmacol.*, 44, 115-128
- Charnley, G. & Tannenbaum, S.R. (1985) A flow cytometric analysis of the effect of sodium chloride on gastric cancer risk in the rat. *Cancer Res.*, 45, 5608-5616
- Charnley, G., Tannenbaum, S.R. & Correa, P. (1982) *Gastric cancer: an etiological model*. In: Magee, P.N., ed., *Nitrosamines and Human Cancer (Banbury Report 12)*, Cold Spring Harbor, NY, CSH Press, pp. 503-522
- The Chemical Daily Co. (1984) *The Specialty Chemicals Handbook*, Tokyo, pp. 309-316
- Chen, J., Campbell, T.C., Li, J. & Peto, R. (1987) An ecologic study on diet and cancer in China. *Natl Cancer Inst. Monogr.* (in press)
- Cheng, S.S. & Li, M.H. (1985) A comparative study on mutagenesis of methylbenzyl nitrosamine in V-79 cells co-cultivated with liver or esophageal epithelial cells from chickens, rats and humans. *Carcinogenesis*, 6, 1731-1737
- Chin, A., Hung, M.-H. & Stock, L.M. (1981) Reactions of benzene-diazonium ions with adenine and its derivatives. *J. org. Chem.*, 46, 2203-2207
- Choi, B.C.K. (1985) *N*-Nitroso compounds and human cancer. A molecular epidemiologic approach. *Am. J. Epidemiol.*, 121, 737-743
- Choi, N.W. (1968) Ethnic distribution of gastrointestinal cancer in Manitoba. *Am. J. publ. Health*, 57, 2067-2081
- Choi, N.W., Entwistle, D.W., Michaluk, W. & Nelson, N.A. (1971) Gastric cancer in Icelanders in Manitoba. *Isr. J. med. Sci.*, 7, 1500-1508
- Chow, T.Y.K. & Fraser, M.J. (1983) Purification and properties of single strand DNA-binding endo-exonuclease of *Neurospora crassa*. *J. biol. Chem.*, 258, 12010-12018

- Chung, F.-L. & Hecht, S.S. (1985) Cyclic 1,N<sup>2</sup>-glyoxal-deoxyguanosine adduct. *Carcinogenesis*, **6**, 1671-1673
- Chung, F.-L., Juchatz, A., Vitarius, J. & Hecht, S.S. (1984) Effects of dietary compounds on  $\alpha$ -hydroxylation of N-nitrosopyrrolidine and N'-nitroso-nornicotine in rat target tissues. *Cancer Res.*, **44**, 2924-2928
- Chung, F.-L., Wang, M. & Hecht, S.S. (1985) Effects of dietary indoles and isothiocyanates on N-nitrosodimethylamine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone  $\alpha$ -hydroxylation and DNA methylation in rat liver. *Carcinogenesis*, **6**, 539-543
- Coleman, M.H. (1978) A model system for the formation of N-nitrosopyrrolidine in grilled or fried bacon. *J. Food Technol.*, **13**, 55-69
- Columbano, A., Ledda, G.M., Rao, P.M., Rajalakshmi, S. & Sarma, D.S.R. (1980) Susceptibility of DMN induced O<sup>6</sup>-methylguanine containing regions in in vivo replicated hybrid rat liver DNA towards S1 nuclease. *Cancer Lett.*, **10**, 333-338
- Combs, G.F. & Clark, L.C. (1985) Can dietary selenium modify cancer risk? *Nutr. Rev.*, **43**, 325-331
- Conrad, J. (1985) *Model Investigations on the Formation and Stability of Nucleotides Alkylated at the Phosphate Group using Trialkylphosphates and Phosphotriesters of oazobenzene (Ger.)*. *Z. physiol Chem.*, **290**, 28-32
- Consbruch, U. & Schmähl, D. (1952) The lactoflavine content in the liver of rats after feeding of 4-dimethylaminoazobenzene (Ger.). *Z. physiol Chem.*, **290**, 28-32
- Cook, A., Hill, M., Hall, N., Northfield, T., Kirkham, J., Viney, N. & Darkin, C. (1985) Gastric juice analyses in patients at high risk of gastric cancer. *Br. J. Cancer*, **52**, 446
- Co-ordinating Group for Research on the Etiology of Esophageal Cancer in North China (1975) The epidemiology of esophageal cancer in north China and preliminary results in the investigation of its etiological factors. *Sci. Sin.*, **18**, 131-148
- Co-ordinating Group for Research on the Etiology of Esophageal Cancer in North China (1977) The preliminary studies on epidemiological factors of esophageal cancer. *Res. Cancer Prev. Treat.*, **2**, 1-8
- Corcoran, G.D., Ware, J., Logan, R. & Gray, S. (1985) The risk of gastric cancer after benign ulcer surgery — a case control study. *Gut*, **26**, A1151, F22
- Correa, P. (1983) The gastric precancerous process. *Cancer Surv.*, **2**, 437-450
- Correa, P., Haenszel, W., Cuello, C., Tannenbaum, S. & Archer, M. (1975) A model for gastric cancer epidemiology. *Lancet*, **ii**, 58-60
- Correa, P., Cuello, C., Fajurdo, L.F., Haenszel, W., Baleros, O. & de Ramirez, B. (1983) Diet and gastric cancer: nutritional survey in a high-risk area. *J. natl Cancer Inst.*, **70**, 673-678
- Correa, P., Fontham, E., Pickle, L.W., Chen, V., Lin, Y. & Haenszel, W. (1985) Dietary determinants of gastric cancer in South Louisiana inhabitants. *J. natl Cancer Inst.*, **75**, 645-654
- Cottrell, R.C., Lake, B.G., Phillips, J.C. & Gangolli, S.D. (1977) The hepatic metabolism of <sup>15</sup>N-labelled dimethylnitrosamine in the rat. *Biochem. Pharmacol.*, **26**, 809-813
- Coughlin, J.R. (1979) *Formation of N-Nitrosamines from Maillard-Browning Reaction Products in the Presence of Nitrite*, PhD dissertation, University of California, Davis, CA, USA
- Coulston, F. & Dunne, J.F. (1980) *An in-vitro test for N-nitrosatability*. In: Coulston, F. & Dunne, J.F., eds, *The Potential Carcinogenicity of Nitrosatable Drugs*, Norwood, NJ, Ablex Publishing Co., p. 8
- Cox, R.D. (1980) Determination of nitrate and nitrite at parts per billion level by chemiluminescence. *Anal. Chem.*, **52**, 323-335
- Craddock, V. (1983) Nitrosamines and human cancer: proof of an association? *Nature*, **306**, 638

- Craddock, V.M. (1984) *Repair and replication of DNA containing O<sup>6</sup>-methylguanine in fetal and adult animal tissues in relation to their susceptibilities to cancer induction by N-nitroso-N-alkylureas*. In: O'Neill, I.K., von Borstel, R.C., Miller, C.T., Long, J. & Bartsch, H., eds, *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer (IARC Scientific Publications No. 57)*, Lyon, International Agency for Research on Cancer, pp. 571-574
- Craddock, V.M. & Henderson, A.R. (1986) Effect of *N*-nitrosamines carcinogenic for oesophagus on O<sup>6</sup>-alkylguanine-DNA-methyl transferase in rat oesophagus and liver. *J. Cancer Res. clin. Oncol.*, *111*, 229-236
- Craddock, V.M. & Magee, P.N. (1967) Effects of administration of the carcinogen dimethylnitrosamine on urinary 7-methylguanine. *Biochem. J.*, *104*, 435-440
- Craddock, V.M., Sparrow, S. & Henderson, A.R. (1986) The effect of the trichothecene mycotoxin diacetoxyscirpenol on nitrosamine-induced esophageal cancer in the rat. *Cancer Lett.*, *31*, 197-204
- Cuello, C., Correa, P., Haenszel, W., Gordillo, G., Brown, C., Archer, M. & Tannenbaum, S. (1976) Gastric cancer in Colombia. I. Cancer risk and suspect environmental agents. *J. natl Cancer Inst.*, *57*, 1015-1020
- Curtius, T. (1904) Spontaneous decomposition of glycolates (Ger.). *Berichte*, *37*, 1284-1300
- Curtius, T. & Thompson, J. (1906) Reaction of ammonia on diazoacetyl glycine ester and diazoacetyl glycyglycine ester (Ger.). *Chem. Ber.*, *39*, 1383-1388
- Czygan, P., Greim, H., Garro, A.J., Hutterer, F., Schaffner, F., Popper, H., Rosenthal, O. & Cooper, D.Y. (1973) Microsomal metabolism of dimethylnitrosamine and the cytochrome P-450 dependency of its activation to mutagen. *Cancer Res.*, *33*, 2983-2986
- Dagani, D. & Archer, M.C. (1976) Deuterium isotope effect in the microsomal metabolism of dimethylnitrosamine. *J. natl Cancer Inst.*, *57*, 955-957; see also correction in *J. natl Cancer Inst.*, *61*, 619
- Dahl, A.R. (1985) *Activation of carcinogens and other xenobiotics by nasal cytochromes P-450*. In: Boobis, A.R., Caldwell, J., DeMatteis, F. & Elcombe, C.R., eds, *Microsomes and Drug Oxidations*, London, Taylor & Francis, pp. 299-309
- Damiani, G., Kiyotaki, C., Soeller, W., Sasada, M., Peisach, J. & Bloom, B.R. (1980) Macrophage variants in oxygen metabolism. *J. exp. Med.*, *152*, 808-822
- Dang Vu, B., Paul, J.L., Ekindjian, O.G., Yonger, J., Gaudric, M. & Guerre, J. (1983) Chemiluminescence of nitrite and *N*-nitroso compounds in gastric juice. *Clin. Chem.*, *29*, 1860-1861
- Dar, M.S. & Bowman, E.R. (1985) In-vivo mammalian metabolism of methylamine and methylurea and their metabolic interrelationship. *Drug Metab. Disposition*, *13*, 682-689
- Daugherty, I.P., Clapp, N.K., Zehfus, M.H. & Brock, S.E. (1977) Radioactive components in the acid-soluble fraction of mouse liver cytosol after dimethylnitrosamine methyl-<sup>14</sup>C administration. *Gann*, *68*, 697-701
- Davies, D.L., Bryant, G.M., Arcos, J.C. & Argus, M.F. (1976) Age-dependence of hepatic dimethylnitrosamine-demethylase activity in the rat. *J. natl Cancer Inst.*, *56*, 1057-1058
- Davies, J.M. (1980) Stomach cancer mortality in Worksop and other Nottinghamshire mining towns. *Br. J. Cancer*, *41*, 438-445
- Davies, R., Dennis, M.J., Massey, R.C. & McWeeny, D.J. (1978) *Some effects of phenol and thiol-nitrosation on N-nitrosamine formation*. In: Walker, E.A., Castegnaro, M., Griciūtė, L. & Lyle, R.E., eds, *Environmental Aspects of N-Nitroso Compounds (IARC Scientific Publications No. 19)*, Lyon, International Agency for Research on Cancer, pp. 183-197
- Day, D.W. (1986) *Biopsy Pathology of the Oesophagus, Stomach and Duodenum*, London, Chapman & Hall
- De Bernardinis, G., Guadagni, S., Pistoia, M.A., Amicucci, G., Masci, C., Agnifili, A. & Carboni, M. (1983) Gastric juice nitrite and bacteria in gastroduodenal disease and resected stomach. *Tumori*, *69*, 231-237

- De Flora, S., Bennicelli, C., Camoirano, A. & Znacchi, P. (1983) Genotoxicity of nitrosated ranitidine. *Carcinogenesis*, *4*, 255-260
- Demple, B., Jacobsson, A., Olsson, M., Robins, P. & Lindahl, T. (1982) Repair of alkylated DNA in *Escherichia coli*. Physical properties of O<sup>6</sup>-methylguanine DNA methyltransferase. *J. biol. Chem.*, *257*, 13776-13780
- Denayer, R. (1962) Syntheses of adenine derivatives substituted in position 3 or in position 7 (Fr.). *Bull. Soc. chim. Fr.*, 1358
- Denkel, E., Pool, B.L., Schlehofer, J.R. & Eisenbrand, G. (1986) Biological activity of N-nitrosodiethanolamine and of potential metabolites which may arise after activation by alcoholdehydrogenase in *Salmonella typhimurium*, in mammalian cells and in vivo. *J. Cancer Res. clin. Oncol.*, *111*, 149-153
- Diaz-Gomez, M.I., Swann, P.F. & Magee, P.N. (1977) The absorption and metabolism in rats of small oral doses of dimethylnitrosamine. Implication for the possible hazard of dimethylnitrosamine in human food. *Biochem. J.*, *164*, 497-500
- Dixon, W.J. (1983) *BMDP, Statistical Software*, Berkeley, CA, University of California Press
- Dodge, J.T., Mitchell, C. & Hanahan, D.J. (1963) The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. *Arch. Biochem. Biophys.*, *100*, 119-130
- Dolan, M.E. & Pegg, A.E. (1985) Extent of formation of O<sup>6</sup>-methylthymidine in calf thymus DNA methylated by N-methyl-N-nitrosourea and lack of repair of this product by rat liver O<sup>6</sup>-alkylguanine-DNA-alkyltransferase. *Carcinogenesis*, *6*, 1611-1614
- Dolan, M.E., Scicchitano, D., Singer, B. & Pegg, A.E. (1984) Comparison of repair of methylated pyrimidines in poly(dT) by extracts from rat liver and *Escherichia coli*. *Biochem. biophys. Res. Commun.*, *123*, 324-330
- Dolan, M.E., Morimoto, K. & Pegg, A.E. (1985a) Reduction of O<sup>6</sup>-alkylguanine-DNA-alkyltransferase activity in HeLa cells treated with O<sup>6</sup>-alkylguanines. *Cancer Res.*, *45*, 6413-6417
- Dolan, M.E., Corsico, C.D. & Pegg, A.E. (1985b) Exposure of HeLa cells to O<sup>6</sup>-alkylguanines increases sensitivity to the cytotoxic effects of alkylating agents. *Biochem. biophys. Res. Commun.*, *132*, 178-185
- Dolan, M.E., Young, G.S. & Pegg, A.E. (1986) The effect of O<sup>6</sup>-alkylguanine pretreatment on the sensitivity of human colon tumor cells to the cytotoxic effects of chloroethylating agents. *Cancer Res.*, *46*, 4500-4504
- Domin, B.A., Serabjit-Singh, C.J., Vanderslice, R.R., Devereux, T.R., Fouts, J.R., Bend, J.R. & Philpot, R.M. (1984) *Tissue and cellular differences in expression of cytochrome P-450 isozymes*. In: Paten, W., Mitchell, J. & Turner, P., eds, *Proceedings of the 9th International Congress of Pharmacology*, London, MacMillan, pp. 219-224
- Domoradzki, J., Pegg, A.E., Dolan, M.E., Maher, V.M. & McCormick, J.J. (1985) Depletion of O<sup>6</sup>-methylguanine-DNA-methyltransferase in human fibroblasts increases the mutagenic response to N-methyl-N'-nitro-N-nitrosoguanidine. *Carcinogenesis*, *6*, 1823-1826
- Dorman, B.H., Siegfried, J. & Kaufman, D.G. (1983) Alterations of human endometrial stromal cells produced by N-methyl-N'-nitro-N-nitrosoguanidine. *Cancer Res.*, *43*, 3348-3357
- Dougherty, R.W., Shuman, R.D., Mullenax, C.H., Witzel, D.A., Buck, W.B., Wood, R.L. & Cook, H.M. (1965) Physiopathological studies of erysipelas in pigs. *Cornell Vet.*, *55*, 87-109
- Douglass, M.L., Kabacoff, B.L., Anderson, G.A. & Cheng, M.C. (1978) The chemistry of nitrosamine formation, inhibition and destruction. *J. Soc. Cosmet. Chem.*, *29*, 581-606
- Doyle, M.P. & Hoekstra, J.W. (1981) Oxidation of nitrogen oxides by bound dioxygen in hemoproteins. *J. inorg. Biochem.*, *14*, 351-358
- Drasar, B.S., Shiner, M. & McLeod, G.M. (1969) Studies on the intestinal flora. I. The bacterial flora of the gastrointestinal tract in healthy and achlorhydric persons. *Gastroenterology*, *56*, 71-79
- Driver, H.E. & McLean, A.E. (1986) Dose-response relationships for initiation of rat liver tumours by diethylnitrosamine and promotion by phenobarbitone or alcohol. *Food Chem. Toxicol.*, *24*, 241-245

- Druckrey, H., Schildbach, A., Schmähl, D., Preussmann, R. & Ivankovic, S. (1963) Quantitative analysis of the carcinogenicity of diethylnitrosamine (Ger.). *Arzneimittelforschung*, *10*, 841-851
- Druckrey, H., Preussmann, R., Ivankovic, S. & Schmähl, D. (1967) Organotropic carcinogenicity of 65 different *N*-nitroso compounds in BD rats (Ger.). *Z. Krebsforsch.*, *69*, 103-201
- Dull, B.J. & Hotchkiss, J.H. (1984a) Nitrate balance and biosynthesis in the ferret. *Toxicol. Lett.*, *23*, 79-89
- Dull, B.J. & Hotchkiss, J.H. (1984b) Activated oxygen and mammalian nitrate biosynthesis. *Carcinogenesis*, *5*, 1161-1164
- Dull, B.J. & Hotchkiss, J.H. (1984c) A rapid method for estimating nitrate in biological samples using gas chromatography with a flame-ionization detector or a thermal energy analyzer. *Food Chem. Toxicol.*, *22*, 105-108
- Dunn, S.R., Pensabene, J.W. & Simenhoff, M.L. (1986) Analysis of human blood for volatile *N*-nitrosamine by gas chromatography-chemiluminescence detection. *J. Chromatogr.*, *377*, 35-47
- Dunham, L.J., Rabson, A.S., Steward, H.L., Frank, A.S. & Young, J.L. (1968) Rates, interview, and pathology study of cancer of the urinary bladder in New Orleans, Louisiana. *J. natl Cancer Inst.*, *41*, 683-709
- Durrant, P.J. & Durrant, B. (1970) *Introduction to Advanced Inorganic Chemistry*, New York, J. Wiley
- Dworkin, B.M., Rosenthal, W.S., Gordon, G.G. & Jankowski, R.H. (1984) Diminished blood selenium levels in alcoholics. *Alcohol. clin. exp. Res.*, *8*, 535-538
- Dyroff, M.C., Richardson, F.C., Popp, J.A., Bedell, M.A. & Swenberg, J.A. (1986) Correlation of *O*-ethyldeoxythymidine accumulation, hepatic initiation and hepatocellular carcinoma induction in rats continuously administered diethylnitrosamine. *Carcinogenesis*, *7*, 247-251
- Edler, L., Wahrendorf, J. & Frank, N. (1983) Computational methods for the screening of pharmacokinetic parameters in metabolism experiments. *Int. J. bio-med. Comput.*, *14*, 369-380
- Eisenbrand, G., Ellen, G., Preussmann, R., Schuller, P.L., Spiegelhalter, B., Stephany, R.W. & Webb, K.S. (1983) *Determination of volatile nitrosamines in food, animal feed and other biological materials by low-temperature vacuum distillation and chemiluminescence detection*. In: Preussmann, R., O'Neill, I.K., Eisenbrand, G., Spiegelhalter, B. & Bartsch, H., eds, *Environmental Carcinogens. Selected Methods of Analysis*, Vol. 6, *N-Nitroso Compounds (IARC Scientific Publications No. 45)*, Lyon, International Agency for Research on Cancer, pp. 181-203
- Eisenbrand, G., Adam, B., Peter, M., Malfertheiner, P. & Schlag, P. (1984a) *Formation of nitrite in gastric juice of patients with various gastric disorders after ingestion of a standard dose of nitrate — a possible risk factor in gastric carcinogenesis*. In: O'Neill, I.K., von Borstel, R.C., Miller, C.T., Long, J. & Bartsch, H., eds, *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer (IARC Scientific Publications No. 57)*, Lyon, International Agency for Research on Cancer, pp. 963-968
- Eisenbrand, G., Denkel, E. & Pool, B. (1984b) Alcoholdehydrogenase as an activating enzyme for *N*-nitrosodiethanolamine (NDELA): in vitro activation of NDELA to a potent mutagen in *Salmonella typhimurium*. *J. Cancer Res. clin. Oncol.*, *108*, 76-80
- Eisenbrand, G., Preussmann, R. & Spiegelhalter, B. (1986) *Vulcanization Accelerators* (German Patent No. 3243141 to German Cancer Research Centre)
- El-Aaser, A.A., El-Merzabani, M.M., Higgy, N.A. & Abdel-Kader, M.M. (1979) A study on the aetiological factors of bilharzial bladder cancer in Egypt. 3. Urinary  $\beta$ -glucuronidase. *Eur. J. Cancer*, *15*, 573-583
- El-Aaser, A.B., El-Merzabani, M.M., El-Bolkainy, M.N., Ibrahim, A.S., Zakhary, N.I. & El-Morsi, B. (1980) A study on the etiological factors of bilharzial bladder cancer in Egypt. 5. Urinary nitrite in a rural population. *Tumori*, *66*, 409-414
- Elgin, S.C.R. (1981) DNase I-hypersensitive sites of chromatin. *Cell*, *27*, 413-415

- Ellmann, G. (1959) Tissue sulfhydryl groups. *Arch. Biochem. Biophys.*, **82**, 70-77
- El-Merzabani, M., El-Aaser, A.B. & Zakhary, N.I. (1979) A study on the aetiological factors of bilharzial bladder cancer in Egypt. I. Nitrosamines and their precursors in urine. *Eur. J. Cancer*, **15**, 287-291
- Elsebai, I. (1978) Cancer of the bilharzial bladder. *Urol. Res.*, **6**, 233-236
- Emura, M., Mohr, U., Kakunaga, T. & Hilfrich, J. (1985) Growth inhibition and transformation of human fetal tracheal epithelial cell line by long-term exposure to diethylnitrosamine. *Carcinogenesis*, **6**, 1079-1085
- Ender, F., Havre, G., Helgebostad, A., Koppang, N., Madsen, R. & Ceh, L. (1964) Isolation and identification of a hepatotoxic factor in herring meal produced from sodium nitrite preserved herring. *Naturwissenschaften*, **51**, 637-638
- Endo, H., Takahashi, K. & Aoyagi, H. (1974) Screening of compounds structurally and functionally related to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, a gastric carcinogen. *Gann*, **65**, 45-54
- Endo, H., Ishizawa, M., Endo, I., Takanashi, K., Utsunomiya, T., Kinoshita, N., Hidaka, K. & Baba, T. (1977) *A possible process of conversion of food components to gastric carcinogens*. In: Hiatt, H.H., Watson, J.D. & Winston, J.A., eds, *Origins of Human Cancer*, Cold Spring Harbor, NY, CSH Press, pp. 1591-1595
- Epstein, J., Rosenthal, R.W. & Ecs, R.J. (1955) Use of gamma-(4-nitrobenzyl)pyridine as analytical reagent for ethylenimines and alkylating agents. *Anal. Chem.*, **27**, 1435-1439
- Eriksson, C., ed. (1981) *Progress in Food and Nutrition Science*, Vol. 5, *Maillard Reactions in Food. Chemical, Physiological and Technical Aspects*, Oxford, Pergamon Press
- Ernster, L., Danielson, L. & Ljunggren, M. (1962) DT diaphorase. I. Purification from the soluble fraction of rat liver cytoplasm and properties. *Biochim. biophys. Acta*, **58**, 171-188
- Fan, T.Y. & Tannenbaum, S.R. (1973) Natural inhibitors of nitrosation reactions: the concept of available nitrite. *J. Food Sci.*, **38**, 1067-1069
- Fan, T.Y., Morrison, J., Rounbehler, D.P., Ross, R., Fine, D.H., Miles, W. & Sen, N.P. (1977a) *N*-Nitrosodiethanolamine in synthetic curing fluids, a part per million impurity. *Science*, **196**, 70-71
- Fan, T.Y., Goff, V., Long, L., Fine, D.H., Arsenault, G.P. & Biemann, K. (1977b) *N*-Nitrosodiethanolamine in consumer cosmetics, lotions and shampoos. *Food Cosmet. Toxicol.*, **15**, 423-430
- Fan, T.Y., Krull, I.S., Ross, R.D., Wolf, M.H. & Fine, D.H. (1978) *Comprehensive analytical procedures for the determination of volatile and non-volatile polar and non-polar N-nitroso compounds*. In: Walker, E.A., Castegnaro, M., Griciūtė, M.L., Lyle, R.E. & Davis, W., eds, *Environmental Aspects of N-Nitroso Compounds (IARC Scientific Publications No. 19)*, Lyon, International Agency for Research on Cancer, pp. 3-17
- Farber, E. & Cameron, R. (1980) The sequential analysis of cancer development. *Adv. Cancer Res.*, **31**, 125-225
- Farmer, P.B., Shuker, D.E.G. & Bird, I. (1986) DNA and protein adducts as indicators of in vivo methylation by nitrosatable drugs. *Carcinogenesis*, **7**, 49-52
- Farrelly, J.G. (1980) A new assay for the microsomal metabolism of nitrosamines. *Cancer Res.*, **40**, 3241-3244
- Farrelly, J.G., Stewart, M.L. & Lijinsky, W. (1984) The metabolism of nitrosodi-*n*-propylamine, nitrosodiallylamine and nitrosodiethanolamine. *Carcinogenesis*, **5**, 1015-1019
- Federal Panel on Formaldehyde (1982) Report of the Federal Panel on Formaldehyde. *Environ. Health Perspect.*, **43**, 139-168
- Fiddler, W., Gates, R.A., Pensabene, J.W., Phillips, J.G. & Wierbicki, E. (1981) Investigations on nitrosamines in irradiation-sterilized bacon. *J. Agric. Food Chem.*, **29**, 551-554



- Fine, D.H. (1978) *Determination of N-nitrosamines in food by chemiluminescence using the TEA*. In: Preussmann, R., Castegnaro, M., Walker, E.A. & Wassermann, A.E. eds, *Environmental Carcinogens. Selected Methods of Analysis*, Vol. 1, *Analysis of Volatile Nitrosamines in Food (IARC Scientific Publications No. 18)*, Lyon, International Agency for Research on Cancer, pp. 133-140
- Fine, D.H. & Rounbehler, D.P. (1975) Trace analysis of volatile N-nitroso compounds by combined gas chromatography and thermal energy analysis. *J. Chromatogr.*, *109*, 271-279
- Fine, D.H., Ross, R., Rounbehler, D.P., Silvergleid, A. & Song, L. (1977) Formation *in vivo* of volatile N-nitrosamine in man after ingestion of cooked bacon and spinach. *Nature*, *265*, 753-755
- Fine, D.H., Challis, B.C., Hartman, P. & Van Ryzin, J. (1982) *Endogenous synthesis of volatile nitrosamines: model calculations and risk assessment*. In: Bartsch, H., O'Neill, I.K., Castegnaro, M. & Okada, M., eds, *N-Nitroso Compounds: Occurrence and Biological Effects (IARC Scientific Publications No. 41)*, Lyon, International Agency for Research on Cancer, pp. 379-396
- Fine, D.H., Rounbehler, D.P., Yu, W.C. & Goff, E.U. (1983) *A new thermal energy analyzer for direct high-performance liquid chromatographic and gas chromatographic analysis of N-nitrosamides*. In: O'Neill, I.K., von Borstel, R.C., Miller, C.T., Long, J. & Bartsch, H., eds, *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer (IARC Scientific Publications No. 57)*, Lyon, International Agency for Research on Cancer, pp. 121-129
- Fishbein, L. (1985) *Core problems in chemical industries: additives used in the plastics, polymer, and rubber industries*. In: Stich, H.F., ed., *Carcinogens and Mutagens in the Environment*, Vol. V, Boca Raton, FL, CRC Press, pp. 75-93
- Fisher, M. & Gordon, T. (1985) The relation of drinking and smoking habits to diet. The lipids research clinics prevalence study. *Am. J. clin. Nutr.*, *41*, 623-630
- Foiles, P.G., Trushin, N. & Castonguay, A. (1985) Measurement of O<sup>6</sup>-methyldeoxyguanosine in DNA methylated by the tobacco specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone using a biotin-avidin enzyme-linked immunosorbent assay. *Carcinogenesis*, *6*, 989-993
- Fong, A.T. & Rasmussen, R.E. (1985) O<sup>6</sup>-Guanine ethylation in target and nontarget cell populations in hamster respiratory tract following acute diethylnitrosamine (DEN) exposure. *Proc. Am. Assoc. Cancer Res.*, *21*, 81
- Fong, L.Y.Y., Sivak, A. & Newberne, P.M. (1978) Zinc-deficiency and methylbenzyl nitrosamine-induced esophageal cancer in rats. *J. natl Cancer Inst.*, *61*, 145-150
- Fong, L.Y.Y., Ng, W.L. & Newberne, P.M. (1984) *N-Nitrosodimethylamine induced forestomach tumours in male Sprague-Dawley rats fed a zinc-deficient diet*. In: O'Neill, I.K., von Borstel, R.C., Miller, C.T., Long, J. & Bartsch, H., eds, *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer (IARC Scientific Publications No. 57)*, Lyon, International Agency for Research on Cancer, pp. 543-546
- Fontham, E., Zavala, D., Correa, P., Rodriguez, E., Hunter, F., Haenszel, W. & Tannenbaum, S.R. (1986) Diet and chronic atrophic gastritis: a case-control study. *J. natl Cancer Inst.*, *76*, 621-627
- Food and Agriculture Organization/World Health Organization (1973) *Energy and Protein Requirements. Report of a Joint FAO/WHO Ad Hoc Expert Committee (World Health Organ. Tech. Rep. Ser. 522)*, Geneva
- Forman, D., Al-Dabbagh, S. & Doll, R. (1985) Nitrates, nitrites and gastric cancer in Great Britain. *Nature*, *313*, 620-625
- Fowler, K.W., Büchi, G. & Essigmann, J.M. (1982) Synthesis and characterization of an oligonucleotide containing a carcinogen-modified base: O<sup>6</sup>-methylguanine. *J. Am. chem. Soc.*, *104*, 1050-1054
- Frank, N. & Wiessler, M. (1983) Alkyldiazohydroxides are stable intermediates in the degradation of N-nitroso-(acetoxymethyl)-alkylamines in rat serum. *Carcinogenesis*, *4*, 751-753
- Frank, N. & Wiessler, M. (1986a) Inhibition of the metabolism of N-nitrosoacetoxymethyl-methylamine in the rat by disulfiram. *Carcinogenesis*, *7*, 755-758

- Frank, N. & Wiessler, M. (1986b) *N*-Nitroso-hydroxyalkyl-alkylamine phosphate esters --- a new class of *N*-nitroso compounds. *Carcinogenesis*, **7**, 365-369
- Frank, N., Hadjiolov, D., Bertram, B. & Wiessler, M. (1980) Effect of disulfiram on the alkylation of rat liver DNA by nitrosodiethylamine. *J. Cancer Res. clin. Oncol.*, **97**, 209-212
- Frei, E., Pool, B.L., Plesch, W.J. & Wiessler, M. (1984) *Biochemical and biological properties of prospective N-nitrodialkylamine metabolites and their derivatives*. In: O'Neill, I.K., von Borstel, R.C., Miller, C.T., Long, J. & Bartsch, H., eds, *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer (IARC Scientific Publications No. 57)*, Lyon, International Agency for Research on Cancer, pp. 491-497
- Frei, E., Pool, B.L., Glatt, H.R., Gemperlin-Mertes, I., Oesch, F., Schlehofer, J.R., Schmezer, P., Weber, H. & Wiessler, M. (1986) Determination of DNA single strand breaks and selective DNA amplification by *N*-nitrodimethylamine and analogs, as well as estimation of the indicator cells' metabolic capacities. *J. Cancer Res. clin. Oncol.*, **111**, 123-128
- Frischer, A. & Ahmad, T. (1977) Severe generalized glutathione reductase deficiency after antitumor treatment with BCNU 1.3-bis-(chloroethyl)-1-nitrosourea. *J. Lab. clin. Chem.*, **89**, 1080-1091
- Fukushima, S., Hagiwara, A., Ogiso, T., Shibata, M. & Ito, N. (1982) Promoting effects of various chemicals in rat urinary bladder carcinogenesis initiated by *N*-nitroso-*n*-butyl-(4-hydroxybutyl)amine. *Food Chem. Toxicol.*, **21**, 59-68
- Furihata, C., Sato, Y., Matsushima, T. & Tatematsu, M. (1985) Induction of ornithine decarboxylase and DNA synthesis in rat stomach mucosa by methylglyoxal. *Carcinogenesis*, **6**, 91-94
- Gaffney, B.L., Marky, L.A. & Jones, R.A. (1984) Synthesis and characterization of a set of four dodecadeoxyribonucleoside undecaphosphates containing *O*<sup>6</sup>-methylguanine opposite adenine, cytosine, guanine and thymine. *Biochemistry*, **23**, 5686-5691
- García, H., Keefer, L., Lijinsky, W. & Wenyon, C.E.M. (1970) Carcinogenicity of nitrosothiomorpholine and 1-nitrosopiperazine in rats. *Z. Krebsforsch.*, **74**, 179-184
- García-Roché, M.O., Bécquer, A. & Arcia, J. (1983) Estimation of the daily intake of nitrates and nitrites which children one to six years old, who attend State Nurseries in the City of Havana, may consume. *Nahrung*, **27**, 583-588
- García-Roché, M.O., Bécquer, A. & Moraleza, N. (1985) Estimation of the daily intake of nitrates and nitrites which children six to eleven years old, who attend primary schools in the City of Havana, may consume. *Nahrung*, **29**, 191-195
- García-Roché M.O., García, A.M. & Torres, O. (1986) Estimate of the daily intake of nitrates and nitrites which students twelve to seventeen years old, who attend secondary boarding schools in the City of Havana, may consume. *Nahrung* (in press)
- Garland, W.A., Kuenzig, W., Rubio, F., Kornychuk, H., Norkus, E.P. & Conney, A.H. (1986) Studies on the urinary excretion of nitrosodimethylamine and nitrosoproline in humans: interindividual and intraindividual differences and the effect of administered ascorbic acid and  $\alpha$ -tocopherol. *Cancer Res.* (in press)
- Garner, R.C. (1985) Assessment of carcinogen exposure in man. *Carcinogenesis*, **6**, 1071-1078
- Gatehouse, D. & Wedd, D. (1983) The bacterial mutagenicity of three naturally occurring indoles after reaction with nitrous acid. *Mutat. Res.*, **124**, 35-51
- Geser, A., Charnay, N., Day, N.E., de-Thé, G. & Ho, J.H.C. (1978) *Environmental factors in the etiology of nasopharyngeal carcinoma: report on a case-control study in Hong Kong*. In: de-Thé, G., Ito, Y. & Davis, W., eds, *Nasopharyngeal Carcinoma: Etiology and Control (IARC Scientific Publications No. 20)*, Lyon, International Agency for Research on Cancer, pp. 213-229
- Gibel, W. (1967) Experimental study of syncarcinogenesis in oesophageal carcinoma (Ger.). *Arch. Geschwulstforsch.*, **30**, 181-189
- Gillatt, P.N., Hart, R.J., Walters, C.L. & Reed, P.I. (1984) Susceptibilities of drugs to nitrosation under standardized chemical conditions. *Food Chem. Toxicol.*, **22**, 269-274

- Gillatt, P.N., Palmer, R.C., Smith, P.L.R., Walters, C.L. & Reed, P.I. (1985) Susceptibilities of drugs to nitrosation under simulated gastric conditions. *Food Chem. Toxicol.*, *23*, 849-855
- Glatt, H.R., Billings, R., Platt, K.L. & Oesch, F. (1981) Improvement of the correlation of bacterial mutagenicity with carcinogenicity of benzo(a)pyrene and four of its major metabolites by activation with intact liver cells instead of cell homogenate. *Cancer Res.*, *41*, 270-277
- Gold, B. & Salmasi, S. (1982) Carcinogenicity tests of acetoxymethylphenylnitrosamine and benzenediazonium tetrafluoroborate in Syrian hamsters. *Cancer Lett.*, *15*, 289-292
- Gold, B., Salamasi, S., Linder, W. & Althoff, J. (1981) Biological and chemical studies involving methyl-*tert*-butylnitrosamine, a noncarcinogenic nitrosamine. *Carcinogenesis*, *2*, 529-532
- Golden, M.H.N., Jackson, A.A. & Golden, B.E. (1977) Effect of zinc on thymus of recently malnourished children. *Lancet*, *ii*, 1057-1059
- Golden, M.H.N., Golden, B.E., Harland, P.S.E.G. & Jackson, A.A. (1978) Zinc and immunocompetence in protein energy malnutrition. *Lancet*, *i*, 1226-1228
- Goldmacher, V.S. & Thilly, W.G. (1983) Formaldehyde is mutagenic for cultured human cells. *Mutat. Res.*, *116*, 417
- Goldstein, E., Pect, N.F., Parks, N.J., Hines, H.H., Steffey, E.O. & Tarkington, B. (1977) Fate and distribution of inhaled nitrogen dioxide in rhesus monkeys. *Am. Rev. resp. Dis.*, *115*, 403-412
- Gombar, C.T., Zubroff, J., Strahan, G.D. & Magee, P.N. (1983) Measurement of 7-methylguanine as an estimate of the amount of dimethylnitrosamine formed following administration of aminopyrine and nitrite to rats. *Cancer Res.*, *43*, 5077-5080
- Gombar, C.T., Pylypiw, H.M. & Harrington, G.W. (1987) Pharmacokinetics of *N*-nitrosodimethylamine in beagles. *Cancer Res.*, *47*, 343-347
- Goodall, C.M., Lijinsky, W., Tomatis, L. & Wenyon, C.E.M. (1970) Toxicity and oncogenicity of nitrosomethylaniline and nitrosomethylcyclohexylamine. *Toxicol. appl. Pharmacol.*, *17*, 426-432
- Gough, T.A., Webb, K.S. & Swann, P.F. (1983) An examination of human blood for the presence of volatile nitrosamines. *Food Chem. Toxicol.*, *21*, 151-156
- Grafström, R.C., Fornace, A.J., Jr & Harris, C.C. (1984) Repair of DNA damage caused by formaldehyde in human cells. *Cancer Res.*, *44*, 4323-4327
- Grafström, R.C., Curren, R.D., Yang, L.L. & Harris, C.C. (1985) Genotoxicity of formaldehyde in cultured human bronchial fibroblasts. *Science*, *228*, 89-91
- Grafström, R.C., Willey, J.C., Sundqvist, K. & Harris, C.C. (1986) *Pathobiological effects of tobacco smoke-related aldehydes in cultured human bronchial epithelial cells*. In: Hoffmann, D. & Harris, C.C., eds, *New Aspects of Tobacco Carcinogenesis (Banbury Report 23)*, Cold Spring Harbor, NY, CSH Press
- Graham, S., Schotz, W. & Martino, P. (1972) Alimentary factors in the epidemiology of gastric cancer. *Cancer*, *30*, 927-938
- Gray, J.I., Irvine, D.M. & Kakuda, Y. (1979) Nitrates and *N*-nitrosamines in cheese. *J. Food Prot.*, *42*, 263-272
- Green, L.C., Wagner, D.A., Ruiz de Luzuriaga, K., Rand, W., Istfan, N., Young, V.R. & Tannenbaum, S.R. (1981) Nitrate biosynthesis in man. *Proc. natl Acad. Sci. USA*, *78*, 7764-7768
- Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S. & Tannenbaum, S.R. (1982) Analysis of nitrate, nitrite, and [<sup>15</sup>N] nitrate in biological fluids. *Anal. Biochem.*, *126*, 131-138
- Green, L.C., Skipper, P.L., Turesky, R.J., Bryant, M.S. & Tannenbaum, S.R. (1984) In vivo dosimetry of 4-aminobiphenyl in rats via a cysteine adduct in hemoglobin. *Cancer Res.*, *44*, 4254-4259
- Green, U. & Althoff, J. (1982) Carcinogenicity of vinyl ethylnitrosamine in Syrian golden hamsters. *J. Cancer Res. clin. Oncol.*, *102*, 227-233

- Greenberg, R.A., Haley, N.Y., Eboel, R.A. & Loda, F.A. (1984) Measuring the exposure of infants to tobacco smoke: nicotine and cotinine in urine and saliva. *New Engl. J. Med.*, *310*, 1075-1078
- Greenblatt, M., Mirvish, S. & So, B.T. (1971) Nitrosamine studies: induction of lung adenomas by concurrent administration of sodium nitrite and secondary amines in Swiss mice. *J. natl Cancer Inst.*, *46*, 1029-1034
- Griciūtė, L., Castegnaro, M. & Béréziat, J.C. (1981) Influence of ethyl alcohol in carcinogenesis with *N*-nitrosodimethylamine. *Cancer Lett.*, *13*, 345-352
- Griciūtė, L., Castegnaro, M. & Béréziat, J.C. (1982) Influence of ethyl alcohol on the carcinogenic activity of *N*-nitrosodi-*n*-propylamine. In: Bartsch, H., O'Neill, I.K., Castegnaro, M. & Okada, M., eds, *N-Nitroso Compounds: Occurrence and Biological Effects (IARC Scientific Publications No. 41)*, Lyon, International Agency for Research on Cancer, pp. 643-648
- Griciūtė, L., Castegnaro, M. & Béréziat, J.C. (1984) Influence of ethyl alcohol on carcinogenesis induced with *N*-nitrosodiethylamine. In: Börzsönyi, M., Day, N.E., Lapis, K. & Yamasaki, H., eds, *Models, Mechanisms and Etiology of Tumour Promotion (IARC Scientific Publications No. 56)*, Lyon, International Agency for Research on Cancer, pp. 413-417
- Grilli, S. & Prodi, G. (1975) Identification of dimethylnitrosoamine metabolites *in vitro*. *Gann*, *66*, 473-480
- Grimmer, G. & Bohnke, H. (1979) *Method 3* and *Method 4*. In: Castegnaro, M., Bogovski, P., Kunte, H. & Walker, E.A., eds, *Environmental Carcinogens: Selected Methods of Analysis*, Vol. 3, *Analysis of Polycyclic Aromatic Hydrocarbons in Environmental Samples (IARC Scientific Publications No. 29)*, Lyon, International Agency for Research on Cancer, pp. 155-162, 163-173
- Gross, R.L., Osdin, N., Fong, L. & Newberne, P.M. (1979) 1. Depressed immunological function in zinc-deprived rats as measured by mitogen response of spleen, thymus, and peripheral blood. *Am. J. clin. Nutr.*, *32*, 1260-1265
- Gruenwedel, D.W., Lynch, S.S. & Russell, G.F. (1984) The influence of 1-(*N*-L-tryptophan)-1-deoxy-D-fructose [FRU-TRP] and its *N*-nitrosated analogue [NO-FRU-TRP] on the viability and intracellular synthetic activity (DNA, RNA, and protein synthesis) of HeLa S3 carcinoma cells. *Adv. exp. Med. Biol.*, *177*, 269-285
- Guengerich, F.P. (1977) Separation and purification of multiple forms of microsomal cytochrome P-450. *J. biol. Chem.*, *252*, 3970-3979
- Gupta, P.C., Mehta, F.S., Daftary, D.K., Pindborg, J.J., Bhonsle, R.B., Jainawalla, P.N., Sinor, P.N., Pitkar, V.K., Murti, P.R., Irani, R.R., Shah, H.T., Kadam, P.M., Iyer, K.S.S., Iyer, H.M., Hedge, A.K., Chandrashekar, G.K., Shroff, B.C., Sahiar, B.E. & Mehta, M.N. (1980) Incidence rates of oral cancer and natural history of oral precancerous lesions in a 10-year follow-up study of Indian villagers. *Commun. dent. oral Epidemiol.*, *8*, 287-333
- Gupta, R.C., Reddy, V.M. & Randerath, K. (1982) 32P-postlabelling analysis of non-radioactive carcinogen-DNA adducts. *Carcinogenesis*, *3*, 1081-1092
- Gurkalo, V.K. & Zabezhinsky, M.A. (1982) Modifying effect of clonidine, caffeine and ethanol on *N*-nitrosodiethylamine-induced carcinogenesis (Russ.). *Eksp. Onkol.*, *4*, 25-29
- Guttenplan, J.B. (1980) Enhanced mutagenic activities of *N*-nitroso compounds in weakly acidic media. *Carcinogenesis*, *1*, 439-444
- Haas, H., Mohr, U. & Kruger, F.W. (1973) Comparative studies with different doses of *N*-nitrosomorpholine, *N*-nitrosopiperidine, *N*-nitrosomethylurea and dimethylnitrosamine in Syrian golden hamsters. *J. natl Cancer Inst.*, *51*, 1295-1301
- Habs, M. & Schmähl, D. (1976) Influence of five different postnatal lifelong treatments on the transplacental carcinogenicity of ethylnitrosourea in Sprague-Dawley rats. *Cancer Lett.*, *2*, 93-100
- Habs, M. & Schmähl, D. (1981) Inhibition of the hepatocarcinogenic activity of diethylnitrosamine (DENa) by ethanol in rats. *Hepato-gastroenterol.*, *28*, 242-244

- Habs, M. & Schmähl, D. (1983) Prevention of urinary bladder tumors in cyclophosphamide-treated rats by additional medication with the uroprotectors sodium 2-mercaptoethane sulfonate (*mesna*) and disodium 2,2'-dithio-bis-ethane sulfonate (*dimesna*). *Cancer*, *51*, 606-609
- Habs, M. & Schmähl, D. (1984) Influence of nicotine on *N*-nitrosomethylurea-induced mammary tumors in rats. *Klin. Wochenschr.*, *62*, 105-108
- Habs, M., Habs, H. & Schmähl, D. (1980) Influence of thioctic acid ( $\alpha$ -lipoic acid) on *N*-nitrosodiethylamine-induced carcinogenesis in male Sprague-Dawley rats. *Arzneimittel-Forsch.*, *30*, 1715-1717
- Habs, M., Schmähl, D. & Lin, P.Z. (1981) Carcinogenic activity in rats of combined treatment with cyclophosphamide, methotrexate and 5-fluorouracil. *Int. J. Cancer*, *28*, 91-96
- Hadley, W.M. & Dahl, A.R. (1982) Cytochrome P-450 dependent monooxygenase activity in rat nasal epithelial membranes. *Toxicol. Lett.*, *10*, 417-422
- Haenszel, W. & Correa, P. (1975) Developments in the epidemiology of stomach cancer over the last decade. *Cancer Res.*, *35*, 3452-3459
- Haenszel, W., Kurihara, M., Segi, M. & Lee, R.K.C. (1972) Stomach cancer among Japanese in Hawaii. *J. natl Cancer Inst.*, *49*, 969-988
- Haenszel, W., Kurihara, M., Locke, F.B., Shimuzu, K. & Segi, M. (1976) Stomach cancer in Japan. *J. natl Cancer Inst.*, *56*, 265-274
- Haenszel, W., Correa, P., Lopez, A., Cuello, C., Zarama, G., Zavala, D. & Fontham, E. (1985) Serum micronutrient levels in relation to gastric pathology. *Int. J. Cancer*, *75*, 43-48
- Haga, J.J., Russell, B.R. & Chapel, J.F. (1972) The kinetics of decomposition of *N*-alkyl derivatives of nitrosoguanidine. *Cancer Res.*, *32*, 2085
- Hagmar, L., Bellander, T., Englander, V., Ranstam, J., Attewell, R. & Skerfving, S. (1986) Mortality and cancer morbidity among workers in a chemical factory. *Scand. J. Work Environ. Health* (in press)
- Hall, C.N., Darkin, D., Brimblecombe, R., Cook, A.J., Kirkham, J.S. & Northfield, T.C. (1986) Evaluation of the nitrosamine hypothesis of gastric carcinogenesis in precancerous conditions. *Gut*, *27*, 491-498
- Hall, J. & Karran, P. (1986) *O*-Methylated pyrimidines — important lesions in cytotoxicity and mutagenicity in mammalian cells. In: Myrnes, B. & Krokan, H., eds, *Repair of DNA Lesions Introduced by N-Nitroso Compounds*, Oslo, Norwegian University Press, pp. 73-88
- Hamm, R. & Hoffmann, K. (1966) Determination of sulphhydryl and disulphide groups in myofibrils and muscle tissue by amperometric titration. *Z. Lebensmitt. Untersuch. Forsch.*, *130*, 133-145
- Hanski, C. & Lawley, P.D. (1985) Urinary excretion of 3-methyladenine and 1-methylnicotinamide by rats following administration of [methyl-<sup>14</sup>C]methyl methanesulphonate and comparison with administration of [<sup>14</sup>C]methionine or formate. *Chem.-biol. Interactions*, *55*, 225-234
- Hard, G.C. (1979) Effect of age at treatment on incidence and type of renal neoplasm induced in the rat by a single dose of dimethylnitrosamine. *Cancer Res.*, *39*, 4965-4970
- Hard, G.C. & Butler, W.H. (1970) Cellular analysis of renal neoplasia: light microscopic study of the development of interstitial lesions induced in the rat kidney by a single carcinogenic dose of dimethylnitrosamine. *Cancer Res.*, *30*, 2806-2815
- Harris, C.C. (1987) Human tissues and cells in carcinogenesis research. *Cancer Res.*, *47*, 1-14
- Harris, C.C., Autrup, H., Stoner, G.D., McDowell, E.M., Trump, B.F. & Schafer, P.W. (1977) Metabolism of acyclic and cyclic *N*-nitrosamines in cultured human bronchi. *J. natl Cancer Inst.*, *59*, 1401-1406
- Harris, C.C., Autrup, H., Stoner, G.D., Trump, B.F., Hillman, E., Schafer, P.W. & Jeffrey, A.M. (1979) Metabolism of benzo[*a*]pyrene, *N*-nitrosodimethylamine and *N*-nitrosopyrrolidine and identification of the major carcinogen-DNA adducts formed in cultured human esophagus. *Cancer Res.*, *39*, 4401-4406

- Harris, C.C., Vahakangas, K., Newman, M.J., Trivers, G.E., Shamsuddin, A., Sinopoli, N., Mann, D. & Wright, W.E. (1985) Detection of benzo[*a*]pyrene diol epoxide-DNA adducts in peripheral blood lymphocytes and antibodies to the adducts in serum from coke oven workers. *Proc. natl Acad. Sci. USA*, *82*, 6672-6676
- Hartman, P.E. (1983) Putative mutagens/carcinogens in foods. I. Nitrate/nitrite ingestion and gastric cancer mortality. *Environ. Mutagenesis*, *5*, 111-121
- Hausmann, H.-J. & Werringloer, J. (1985) Oxidative denitrosation and activation of *N*-nitrosodimethylamine. *Biochem. Pharmacol.*, *34*, 411-412
- Haverkos, H.W., Pinsky, P.F., Drotman, D.P. & Bregman, D.J. (1985) AIDS, nitrite and Kaposi's sarcoma. *Sex. Transm. Dis.*, *12*, 203-208
- Havery, D.C. & Fazio, T. (1982) Estimation of volatile *N*-nitrosamines in rubber nipples for babies' bottles. *Food Chem. Toxicol.*, *20*, 939-944
- Havery, D.C. & Fazio, T. (1985) Human exposure to nitrosamines from foods. *Food Technol.*, *39*, 80-83
- Hawksworth, G.M. & Hill, M.J. (1971) Bacteria and the *N*-nitrosation of secondary amines. *Br. J. Cancer*, *25*, 520-526
- Hawksworth, G. & Hill, M.J. (1974) The in vivo formation of *N*-nitrosamines in the rat bladder and their subsequent absorption. *Br. J. Cancer*, *29*, 353-358
- Hayatsu, H., Shimada, H. & Arimoto, S. (1984) Activation of promutagenic *N*-nitrosomorpholine and other *N*-nitrosoalkylamines by near-ultraviolet irradiation in the presence of phosphates. *Gann*, *75*, 203-206
- Haynes, D.C., Gershwin, M.E., Golub, M.S., Cheung, A.T.W., Hurley, L.S. & Hendrickx, A.G. (1985) Studies of marginal zinc deprivation in rhesus monkeys: VI. Influence on the immunohematology of infants in the first year. *Am. J. clin. Nutr.*, *42*, 252-262
- Hazell, S.L. & Lee, A. (1986) *Campylobacter pyloridis*, urease, hydrogen ion back diffusion and gastric ulcers. *Lancet*, *ii*, 15-17
- Heath, D.F. (1962) The decomposition and toxicity of dialkylnitrosamines in rats. *Biochem. J.*, *85*, 72-91
- Heath, D.F. & Dutton, A. (1958) The detection of metabolic products from dimethylnitrosamine in rats and mice. *Biochem. J.*, *70*, 619-626
- Hecht, S.S. (1984) *N*-Nitroso-2-hydroxymorpholine, a mutagenic metabolite of *N*-nitrosodiethanolamine. *Carcinogenesis*, *5*, 1745-1747
- Hecht, S.S. & Morrison, B. (1984) A sensitive method for detecting in vivo formation of *N*-nitrosomorpholine and its application to rats given low doses of morpholine and sodium nitrite. *Cancer Res.*, *44*, 2873-2877
- Hecht, S.S., Chen, C.B., Dong, M., Orna, M. & Hoffmann, D. (1977) Studies on non-volatile nitrosamines in tobacco. *Beitr. Tabakforsch.*, *9*, 1-6
- Hecht, S.S., Chen, C.B., Ohmori, T. & Hoffmann, D. (1980a) Comparative carcinogenicity in F344 rats of the tobacco-specific nitrosamines, *N'*-nitrososornicotine and 4-(*N*-methyl-*N*-nitrosamino)-1-butanone. *Cancer Res.*, *40*, 298-302
- Hecht, S.S., Young, R. & Chen, C.B. (1980b) Metabolism in the F344 rat of 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone, a tobacco-specific carcinogen. *Cancer Res.*, *40*, 4144-4150
- Hecht, S.S., Castonguay, A., Chung, F.-L. & Hoffmann, D. (1982a) *Recent studies on the metabolic activation of cyclic nitrosamines*. In: Magee, P.N., ed., *N-Nitroso Compounds (Banbury Report 12)*, Cold Spring Harbor, NY, CSH Press, pp. 103-120
- Hecht, S.S., Reiss, B., Lin, D. & Williams, G.M. (1982b) Metabolism of *N'*-nitrososornicotine by cultured rat esophagus. *Carcinogenesis*, *3*, 453-456

- Hecht, S.S., Castonguay, A., Chung, F.-L. & Hoffmann, D. (1984) *Carcinogenicity and metabolic activation of tobacco-specific nitrosamines: current status and future prospects*. In: O'Neill, I.K., von Borstel, R.C., Miller, C.T., Long, J. & Bartsch, H., eds, *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer (IARC Scientific Publications No. 57)*, Lyon, International Agency for Research on Cancer, pp. 763-778
- Hecht, S.S., Trushin, N., Castonguay, A. & Rivenson, A. (1986a) Comparative carcinogenicity and DNA methylation in F344 rats by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and *N*-nitrosodimethylamine. *Cancer Res.*, *46*, 498-502
- Hecht, S.S., Lin, D., Chuang, J. & Castonguay, A. (1986b) Reactions with deoxyguanosine of 4-(carbethoxynitrosamino)-1-(3-pyridyl)-1-butanone, a model compound for  $\alpha$ -hydroxylation of tobacco specific nitrosamines. *J. Am. chem. Soc.*, *108*, 1292-1295
- Hecht, S.S., Rivenson, A., Braley, J., DiBello, J., Adams, J.D. & Hoffmann, D. (1986c) Induction of oral cavity tumors in F344 rats by tobacco-specific nitrosamines and snuff. *Cancer Res.*, *4*, 4162-4166
- Hecker, E. (1978) Co-carcinogenic and conditionally cancer-causing factors: new aspects of the etiology of human cancers and molecular mechanisms in carcinogenesis (Ger.) *Naturwissenschaften*, *65*, 640-648
- Helgason, T., Ewen, S.W.B., Jaffray, B., Stowers, J.M., Outram, J.R. & Pollock, J.R.A. (1984) *N-Nitrosamines in smoked meats and their relation to diabetes*. In: O'Neill, I.K., von Borstel, R.C., Miller, C.T., Long, J. & Bartsch, H., eds, *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer (IARC Scientific Publications No. 57)*, Lyon, International Agency for Research on Cancer, pp. 911-920
- Hemminki, K. (1981) Reactions of formaldehyde with guanosine. *Toxicol. Lett.*, *9*, 161-169
- Hemminki, K. (1982) Dimethylnitrosamine adducts excreted in rat urine. *Chem.-biol. Interactions*, *39*, 133-148
- Henbest, H.B., Jones, E.R.H. & Smith, G.F. (1953) Isolation of a new plant-growth hormone, 3-indolylacetonitrile. *J. chem. Soc.*, 3796-3801
- Henderson, B.E. & Louie, E. (1978) *Discussion of risk factors for nasopharyngeal carcinoma*. In: de-Thé, G. & Ito, Y., eds, *Nasopharyngeal Carcinoma: Etiology and Control (IARC Scientific Publications No. 20)*, Lyon, International Agency for Research on Cancer, pp. 251-260
- Herrold, K.M. & Dunham, L.J. (1963) Induction of tumours in the Syrian hamster with diethylnitrosamine (*N*-nitrosodiethylamine). *Cancer Res.*, *23*, 713-777
- Herron, D.C. & Shank, R.C. (1979) Quantitative high pressure liquid chromatographic analysis of methylated purines in rats treated with chemical carcinogens. *Anal. Biochem.*, *100*, 58-63
- Herron, D.C. & Shank, R.C. (1980) Methylated purines in human liver DNA after probable dimethylnitrosamine poisoning. *Cancer Res.*, *40*, 3116-3117
- Heydt, G., Eisenbrand, G. & Preussmann, R. (1982) Metabolism of carcinogenic and non-carcinogenic *N*-nitroso-*N*-methylaminopyridines. I. Investigations *in vitro*. *Carcinogenesis*, *3*, 445-448
- Heydt-Zapf, G., Haubner, R., Preussmann, R. & Eisenbrand, G. (1983) Metabolism of carcinogenic and non-carcinogenic *N*-nitroso-*N*-methylaminopyridines. II. Investigations *in vivo*. *Carcinogenesis*, *4*, 729-731
- Heyns, K. & Beck, M. (1957) The synthesis of D-galactose amino acid (2-amino-2-deoxy-D-galacturon acid) (Ger.). *Chem. Ber.*, *90*, 2443-2447
- Heyns, K., Röper, S., Röper, H. & Meyer, B. (1979) *N*-Nitroso sugar amino acids. *Angew. Chem. int. Ed. Engl.*, *18*, 878-880
- Hicks, R.M. (1980) Multistage carcinogenesis in the urinary bladder. *Br. med. Bull.*, *36*, 39-46

- Hicks, R.M., Walters, C.L., Elsebai, I., El-Aaser, A.B., El-Merzabani, M. & Gough, T.A. (1977) Demonstration of nitrosamines in human urine: preliminary observations on a possible etiology for bladder cancer in association with chronic urinary tract infections. *Proc. R. Soc. Med.*, *70*, 413-416
- Hicks, R.M., Ismail, M.M., Walters, C.L., Beecham, P.T., Rabie, M.T. & El-Alamy, M.A. (1982) Association of bacteriuria and urinary nitrosamine formation with *Schistosoma haematobium* infection in the Qalyub area of Egypt. *Trans. R. Soc. trop. Med. Hyg.*, *76*, 519-527
- Higginson, J. (1966) Etiological factors in gastrointestinal cancer in man. *J. natl Cancer Inst.*, *37*, 527-545
- Hill, M.J., Hawksworth, G. & Tattersall, G. (1973) Bacteria, nitrosamines and cancer of the stomach. *Br. J. Cancer*, *28*, 562-567
- Hinds, M.W. & Kolonel, L.N. (1984) Dietary vitamin A, carotene, vitamin C and risk of lung cancer in Hawaii. *Am. J. Epidemiol.*, *119*, 227-237
- Hirayama, T. (1966) An epidemiological study of oral and pharyngeal cancer in Central and South East Asia. *Bull. World Health Organ.*, *34*, 41-69
- Hirayama, T. (1971) Epidemiology of stomach cancer. *Gann Monogr. Cancer Res.*, *11*, 3-19
- Hirayama, T. (1979) *The epidemiology of gastric cancer in Japan*. In: Pfeiffer, C.J., ed., *Gastric Cancer*, New York, Gerhard Witzstrock, pp. 60-82
- Hirayama, T. (1981) The epidemiology of cancer of the stomach in Japan, with special reference to the role of diet. *Gann Monogr.*, *3*, 15-27
- Hirayama, T. (1984) Cancer mortality in nonsmoking women with smoking husbands based on a large scale cohort study in Japan. *Prev. Med.*, *13*, 680-690
- Ho, J.C.I., Wu, P.C. & Mak, T.K. (1981) Liver cell dysplasia in association with hepatocellular carcinoma, cirrhosis and hepatitis B surface antigen in Hong Kong. *Int. J. Cancer*, *28*, 571-574
- Ho, J.H.C. (1971) *Genetic and environmental factors in nasopharyngeal carcinoma*. In: Nakahara, W., Nishioka, K., Hirayama, T. & Ito, Y., eds, *Recent Advances in Human Tumor Virology and Immunology*, Tokyo, Tokyo University Press, pp. 275-295
- Ho, J.H. (1978) An epidemiologic and clinical study of nasopharyngeal carcinoma. *Int. J. Radiat. Oncol. Biol. Phys.*, *4*, 183-198
- Ho, J.H.C. (1979) Some epidemiologic observations on cancer in Hong Kong. *Natl Cancer Inst. Monogr.*, *53*, 35-47
- Hoar, R.M. (1984) Use of ferrets *Mustela putorius furo* in toxicity testing. *J. Am. Coll. Toxicol.*, *6*, 325-330
- Hodge, J.E. (1953) chemistry of browning reactions in model systems. *J. Agric. Food Chem.*, *1*, 928-943
- Hodgson, R.M., Wiessler, M. & Kleihues, P. (1980) Preferential methylation of target organ DNA by the oesophageal carcinogen *N*-nitrosomethylbenzylamine. *Carcinogenesis*, *1*, 861-866
- Hodgson, R.M., Schwensberg, F., Wiessler, M. & Kleihues, P. (1982) Mechanism of esophageal tumor induction in rats by *N*-nitrosomethylbenzylamine and its ring-methylated analog *N*-nitrosomethyl(4-methylbenzyl)amine. *Cancer Res.*, *42*, 2836-2840
- Hoey, J., Martvern, C. & Lambert, R. (1981) Wine and tobacco: risk factors for gastric cancer in France. *Am. J. Epidemiol.*, *113*, 668-674
- von Hofe, E. & Kleihues, P. (1986) Comparative studies on hepatic DNA alkylation by *N*-nitrosomethylethylamine and *N*-nitrosodimethylamine plus *N*-nitrosodiethylamine. *J. Cancer Res. Clin. Oncol.* (in press)
- von Hofe, E., Grahmann, F., Keefer, L.K., Lijinsky, W., Nelson, V. & Kleihues, P. (1986a) Methylation versus ethylation of DNA in target and nontarget tissues of Fischer 344 rats treated with *N*-nitrosomethylethylamine. *Cancer Res.*, *46*, 1038-1042
- von Hofe, E., Kleihues, P. & Keefer, L.K. (1986b) Extent of DNA 2-hydroxylation by *N*-nitrosomethylethylamine and *N*-nitrosodiethylamine. *Carcinogenesis*, *7*, 1335-1337
- Hoffmann, D. & Adams, J.D. (1981) Carcinogenic tobacco-specific *N*-nitrosamines in snuff and in the saliva of snuff-dippers. *Cancer Res.*, *41*, 4305-4308



- Hoffmann, D. & Brunnemann, K. (1983) Endogenous formation of *N*-nitrosoproline in cigarette smokers. *Cancer Res.*, *43*, 5570-5574
- Hoffmann, D. & Hecht, S.S. (1985) Nicotine-derived *N*-nitrosamines and tobacco related cancer: current status and future directions. *Cancer Res.*, *45*, 935-942
- Hoffmann, D., Raineri, R., Hecht, S.S., Maronpot, R. & Wynder, E.L. (1975) A study of tobacco carcinogenesis. XIV. Effects of *N'*-nitrosornicotine and *N'*-nitrosoanabasine in rats. *J. natl Cancer Inst.*, *55*, 977-981
- Hoffmann, D., Hecht, S.S., Ornaf, R.M., Tso, T.C. & Wynder, E.L. (1976) *Chemical studies on tobacco smoke. XLII. N'-Nitrosornicotine: presence in tobacco, formation and carcinogenicity.* In: Walker, E.A., Bogovski, P. & Griciūtė, L., eds, *Environmental N-Nitroso Compounds. Analysis and Formation (IARC Scientific Publications No. 14)*, Lyon, International Agency for Research on Cancer, pp. 307-320
- Hoffmann, D., Adams, J.D., Brunnemann, K.D. & Hecht, S.S. (1979) Assessment of tobacco-specific *N*-nitrosamines in tobacco products. *Cancer Res.*, *39*, 2505-2509
- Hoffmann, D., Castonguay, A., Rivenson, A. & Hecht, S.S. (1981) Comparative carcinogenicity and metabolism of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and *N'*-nitrosornicotine in Syrian golden hamsters. *Cancer Res.*, *41*, 2386-2393
- Hoffmann, D., Brunnemann, K.D., Adams, J.D. & Hecht, S.S. (1984a) *Formation and analysis of N-nitrosamines in tobacco products and their endogenous formation in consumers.* In: O'Neill, I.K., von Borstel, R.C., Miller, C.T., Long, J. & Bartsch, H., eds, *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer (IARC Scientific Publications No. 57)*, Lyon, International Agency for Research on Cancer, pp. 743-762
- Hoffmann, D., Haley, N.J., Adams, J.D. & Brunnemann, K.D. (1984b) Tobacco sidestream smoke: uptake by non-smokers. *Prev. Med.*, *13*, 608-617
- Hoffmann, D., Rivenson, A., Amin, J. & Hecht, S.S. (1984c) Dose-response study of the carcinogenicity of tobacco specific *N*-nitrosamines in F344 rats. *J. Cancer Res. clin. Oncol.*, *108*, 81-86
- Hoffmann, D., Brunnemann, K.D., Adams, J.D. & Hecht, S.S. (1986a) Laboratory studies on snuff-dipping and oral cancer. *Cancer J.*, *1*, 10-13
- Hoffmann, D., Harley, N.H., Fisenne, I., Adams, J.D. & Brunnemann, K.D. (1986b) Carcinogenic agents in snuff. *J. natl Cancer Inst.*, *76*, 435-437
- Hollander, M. & Wolfe, D.A. (1973) *Non-parametric Statistical Methods*, New York, John Wiley
- Hollenberg, P.F., Miwa, G.T., Walsh, J.S., Dwyer, L.A., Rickert, D.E. & Kedderis, G.L. (1985) Mechanisms of *N*-dimethylation reactions catalyzed by cytochrome P-450 and peroxidases. *Drug Metab. Dispos.*, *13*, 272-275
- Hong, J. & Yang, C.S. (1985) The nature of microsomal *N*-nitrosodimethylamine demethylase and its role in carcinogen activation. *Carcinogenesis*, *6*, 1805-1809
- Horie, S., Kohchi, S. & Kuratsune, M. (1965) Carcinogenesis in the oesophagus. II. Experimental production of oesophageal cancer by administration of ethanolic solutions of carcinogens. *Gann*, *56*, 429-441
- Horn, D. (1982) *Polyethyleneimine — physicochemical properties and applications.* In: Goethals, E.J., ed., *Polymeric Amines and Ammonium Salts*, Oxford, Pergamon Press, pp. 333-355
- Howe, G.R., Burch, J.D., Miller, A.B., Cook, G.M., Estève, J., Morrison, B., Gordeon, P., Chambers, L.W., Fodor, G. & Winsor, G.M. (1980) Tobacco use, occupation, coffee, various nutrients and bladder cancer. *J. natl Cancer Inst.*, *64*, 701-713
- Howell, J.R. (1985) *The Formation and Metabolism of N-7-Carboxymethylguanine*, M.Sc. Thesis, University of Surrey, Guildford, Surrey, UK
- Huang, D.P., Ho, J.H.C., Gough, T.A. & Webb, K.S. (1977) Volatile nitrosamines in some traditional Chinese food products. *J. Food Saf.*, *1*, 1-6

- Huang, D.P., Ho, J.H.C. & Gough, T.A. (1978a) *Analysis for volatile nitrosamines in salt-preserved foodstuffs traditionally consumed by southern Chinese*. In: de-Thé, G. & Ito, Y., eds, *Nasopharyngeal Carcinoma: Etiology and Control (IARC Scientific Publications No. 20)*, Lyon, International Agency for Research on Cancer, pp. 309-314
- Huang, D.P., Ho, J.H.C., Saw, D. & Teoh, T.B. (1978b) *Carcinoma of the nasal and paranasal regions in rats fed Cantonese salted marine fish*. In: de-Thé, G. & Ito, Y., eds, *Nasopharyngeal Carcinoma: Etiology and Control (IARC Scientific Publications No. 20)*, Lyon, International Agency for Research on Cancer, pp. 315-328
- Huang, D.P., Ho, J.H.C., Webb, K.S., Wood, B.J. & Gough, T.A. (1981) Volatile nitrosamines in salt-preserved fish before and after cooking. *Food Cosmet. Toxicol.*, 19, 167-171
- Huang, M., Wang, X., Wang, Z., Zhou, C. & Wu, M. (1986) The study of malignant transformation of human epithelial cells induced by diethylnitrosamine in vitro. *Sci. Sin.* (in press)
- Huber, K.W. & Lutz, W.K. (1984) Methylation of DNA in stomach and small intestine of rats after oral administration of methylamine and nitrite. *Carcinogenesis*, 5, 1729-1732
- Hubert, A. & de-Thé, G. (1986) Nasopharyngeal carcinoma: an anthropological approach to environmental factors (submitted for publication)
- Hung, M.-H. & Stock, L.M. (1982) Reactions of benzenediazonium ions with guanine and its derivatives. *J. org. Chem.*, 47, 448-453
- IARC (1978a) *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans*, Vol. 17, *Some N-Nitroso Compounds*, Lyon, pp. 263-280
- IARC (1978b) *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans*, Vol. 17, *Some N-Nitroso Compounds*, Lyon, pp. 125-175, 313-326
- IARC (1979) *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans*, Vol. 21, *Sex Hormones (II)*, Lyon, pp. 147-364
- IARC (1985) *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans*, Vol. 37, *Tobacco Habits other than Smoking; Betel-quid and Areca-nut Chewing; and some Related Nitrosamines*, Lyon
- IARC (1986) *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans*, Vol. 38, *Tobacco Smoking*, Lyon
- Igarashi, T., Satoh, T., Ueno, K. & Kitagawa, H. (1983) Sex-related difference in the hepatic glutathione level and related enzyme activities in rat. *J. Biochem.*, 93, 33-36
- Ingledew, J.W. & Poole, R.K. (1984) The respiratory chains of *Escherichia coli*. *Microbiol. Rev.*, 48, 222-270
- International Organization for Standardization (1975a) *Meats and Meat Products — Determination of Nitrate Content (ISO 3901)*, Geneva
- International Organization for Standardization (1975b) *Meats and Meat Products — Determination of Nitrite Content (ISO 2918)*, Geneva
- Ireland, C.B., Hytrek, F.P. & Lasoski, B.A. (1980) Aqueous extraction of *N*-nitrosamines from elastomers. *Am. ind. Hyg. Assoc. J.*, 41, 895-900
- Ishiwata, H., Mizushiro, H., Sakai, A. & Tanimura, A. (1981) Distribution, secretion and metabolism of nitrate in the rat lower digestive tract. IV. Metabolic fate of the precursors of *N*-nitroso compounds. *J. Food Hyg. Soc. Jpn.*, 22, 520-525
- Issenberg, P., Conrad, E., Nielson, J., Klein, D. & Miller, S.E. (1984) *Determination of N-nitrosobis(2-hydroxypropyl)amine in environmental samples*. In: O'Neill, I.K., von Borstel, R.C., Miller, C.T., Long, J. & Bartsch, H., eds, *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer (IARC Scientific Publications No. 57)*, Lyon, International Agency for Research on Cancer, pp. 43-50
- Ivankovic, S., Zeller, W.J. & Schmähl, D. (1972) Increased carcinogenic effect of ethylnitrosourea by heavy metals (Ger.). *Naturwissenschaften*, 59, 369

- Ivankovic, S., Preussmann, R., Schmähl, D. & Zeller, W.J. (1974) *Prevention by ascorbic acid of in vivo formation of N-nitroso compounds*. In: Bogovski, P. & Walker, E.A., eds, *N-Nitroso Compounds in the Environment (IARC Scientific Publications No. 9)*, Lyon, International Agency for Research on Cancer, pp. 101-102
- Iversen, O.H. (1980) Tumorigenicity of N-nitroso-diaethyl-, -dimethyl and -diphenyl-amines in skin painting experiments. *Eur. J. Cancer*, *16*, 695-698
- Jain, M., Howe, G.R., Johnson, K.C. & Miller, A.B. (1980) Evaluation of a diet history questionnaire for epidemiologic studies. *Am. J. Epidemiol.*, *111*, 212-219
- Janicek, M.F., Haseltine, W.A. & Henner, W.D. (1985) Malondialdehyde precursors in gamma-irradiated DNA, deoxynucleotides and deoxynucleosides. *Nucleic Acids Res.*, *13*, 9011-9029
- Janzowski, C., Gottfried, J., Eisenbrand, G. & Preussmann, R. (1982a) Fluoro-substituted N-nitrosamines. 3. Microsomal metabolism of N-nitrosodibutylamine and of fluorinated analogs. *Carcinogenesis*, *3*, 777-780
- Janzowski, C., Pool, B.L., Preussmann, R. & Eisenbrand, G. (1982b) Fluoro-substituted N-nitrosamines. 2. Metabolism of N-nitrosodiethylamine and of fluorinated analogs in liver microsomal fractions. *Carcinogenesis*, *3*, 155-159
- Jedrychowski, W., Poliela, T., Tobiasz-Adamczyk, B. & Grzesik, S. (1980) Dietary factors and laxatives in stomach cancer epidemiology (Pol.). *Nowotwory*, *30*, 353-360
- Jefcoate, C.R. (1978) Measurement of substrate and inhibitor binding to microsomal cytochrome P-450 by optical-difference spectroscopy. *Meth. Enzymol.*, *52*, 258-279
- Jennings, W. & Mehran, M.F. (1986) Sample injection in gas chromatography. *J. chromatogr. Sci.*, *24*, 34-40
- Jensen, D.E. & Reed, D.J. (1978) Reaction of DNA with alkylating agents. Quantitation of alkylation by ethylnitrosourea of oxygen and nitrogen sites on poly[dA-dT] including phosphotriester formation. *Biochemistry*, *17*, 5098-5107
- Jensen, D.E., Lotlikar, P.D. & Magee, P.N. (1981) The in vitro methylation of DNA by microsomally-activated dimethylnitrosamine and its correlation with formaldehyde production. *Carcinogenesis*, *21*, 349-354
- Jewell, D.P., Katiyar, V.N., Rees, C., Taylor, K.B. & Wright, J.P. (1975) Isolation of parietal cells from guinea-pig gastric mucosa and the immunological characterization of their antigenic structure. *Gut*, *16*, 603-612
- Johnson, R.A., Jr, Rounbehler, D.P. & Fine, D.H. (1986a) Nitrosamide detection with a TEA analyzer. *Anal. Chem.* (submitted)
- Johnson, R.A., Jr, Goff, E.U. & Fine, D.H. (1986b) Analysis of nitrosourea anticancer drugs with the TEA. *Anal. Chem.* (submitted)
- Johnston, R.B., Jr, Godzik, C.A. & Cohn, Z.A. (1978) Increased superoxide anion production by immunologically activated and chemically elicited macrophages. *J. exp. Med.*, *148*, 115-127
- Jones, F.E., Komorowski, R.A. & Condon, R.E. (1981) Chemoprevention of 1,2-dimethylhydrazine-induced large bowel neoplasms. *Surg. Forum*, *32*, 435-437
- Jones, K.G., Holland, J.F., Foureman, G.L., Bend, J.R. & Fouts, J.R. (1983) Xenobiotic metabolism in Clara cells and alveolar type II cells isolated from lungs of rats treated with  $\beta$ -naphthoflavone. *J. Pharmacol. exp. Ther.*, *225*, 316-319
- Joossens, J.V. & Geboers, J. (1981) Nutrition and gastric cancer. *Nutr. Cancer.*, *2*, 250-261
- Juha'sz, L. (1980) *Descriptive epidemiology of cancer in County Szabolcz-Szatmar, Hungary*. In: Ichikawa, H., ed., *The Present State of Diagnosis and Treatment of Cancer (WHO-CC Monograph 3)*, Tokyo, World Health Organization, pp. 57-71
- Juha'sz, L., Hill, M.J. & Nagy, G. (1980) *Possible relationship between nitrate in drinking water and incidence of stomach cancer*. In: Walker, E.A., Castegnaro, M., Griciūtė, L. & Börzsönyi, M., eds, *N-Nitroso Compounds: Analysis, Formation and Occurrence (IARC Scientific Publications No. 31)*, Lyon, International Agency for Research on Cancer, pp. 619-623
- Jussawalla, D.J. & Deshpande, V.A. (1971) Evaluation of cancer risk in tobacco chewers and smokers. *Cancer*, *28*, 244-252

- Kadlubar, F.F., Miller, J.A. & Miller, E.C. (1977) Hepatic microsomal *N*-glucuronidation and nucleic acid binding of *N*-hydroxy arylamines in relation to urinary bladder carcinogenesis. *Cancer Res.*, **37**, 805-814
- Kahl, R. (1984) Synthetic antioxidants: biochemical actions and interference with radiation, toxic compounds, chemical mutagens and chemical carcinogens. *Toxicology*, **33**, 185-228
- Kamiyama, S. & Michioka, O. (1983) *Mutagenic components of diets in high- and low-risk areas for stomach cancer*. In: Stich, H.F., ed., *Carcinogens and Mutagens in the Environment*, Vol. 3, *Naturally Occurring Compounds: Epidemiology and Distribution*, Boca Raton, FL, CRC Press, pp. 29-42
- Kaneko, M., Nagata, C. & Kodama, M. (1985) Repair of indirectly induced DNA damage in human skin fibroblasts treated with *N*-hydroxy-2-naphthylamine. *Mutat. Res.*, **143**, 103-108
- Kantor, A.F., Hartge, P., Hoover, R.N., Narayana, A.S., Sullivan, J.W. & Fraumeni, J.F., Jr (1984) Urinary tract infection and risk of bladder cancer. *Am. J. Epidemiol.*, **119**, 510-515
- Karran, P. (1985) Possible depletion of a DNA repair enzyme in human lymphoma cells by subversive repair. *Proc. natl Acad. Sci. USA*, **82**, 5285-5289
- Karran, P. & Williams, S.A. (1985) The cytotoxic and mutagenic effects of alkylating agents on human lymphoid cells are caused by different DNA lesions. *Carcinogenesis*, **6**, 789-792
- Kawabata, T., Ohshima, H., Uibu, J., Nakamura, M., Matsui, M. & Hamano, M. (1979) *Occurrence, formation and precursors of N-nitroso compounds in Japanese diet*. In: Miller, E.C., Miller, J.A., Hirono, I., Sugimura, T. & Takayama, S., eds, *Naturally Occurring Carcinogens-Mutagens and Modulators of Carcinogenesis*, Tokyo, Japan Scientific Society Press/Baltimore, University Park Press, pp. 195-209
- Kawamura, T., Sakai, K., Miyazawa, F., Wada, H., Ito, Y. & Tanimura, A. (1971a) Studies on nitrosamines in foods. IV. Distribution of secondary amines in foods. *J. Food Hyg. Soc. Jpn*, **12**, 192-197
- Kawamura, T., Sakai, K., Miyazawa, F., Wada, H., Ito, Y. & Tanimura, A. (1971b) Studies on nitrosamines in foods. V. Distribution of secondary amines in foods. *J. Food Hyg. Soc. Jpn*, **12**, 394-398
- Kawanishi, T., Ohno, Y., Takahashi, A., Takanaka, A., Kasuya, Y. & Omori, Y. (1985a) Substrate concentration dependency of *N*-nitrosodimethylamine and *N*-nitrosomethylbenzylamine metabolism in rat liver. *Carcinogenesis*, **6**, 823-828
- Kawanishi, T., Ohno, Y., Takahashi, A., Takanaka, A., Kasuya, Y. & Omori, Y. (1985b) Relation between hepatic microsomal metabolism of *N*-nitrosamines and cytochrome P-450 species. *Biochem. Pharmacol.*, **34**, 919-924
- Keefer, L.K. & Roller, P.R. (1973) *N*-Nitrosation by nitrite ion in neutral and basic medium. *Science*, **181**, 1245-1247
- Keefer, L.K., Lijinsky, W. & Garcia, H. (1973) Deuterium isotope effect on the carcinogenicity of dimethylnitrosamine in rat liver. *J. natl Cancer Inst.*, **51**, 299-302
- Keefer, L.K., Garland, W.A., Oldfield, N.F., Swagzdis, J.E. & Mico, B.A. (1985) Inhibition of *N*-nitrosodimethylamine metabolism in rats by ether anesthesia. *Cancer Res.*, **45**, 5457-5460
- Keefer, L.K., Anjo, T., Wade, D., Wang, T. & Yang, C.S. (1987) Concurrent generation of methylamine and nitrite during denitrosation of *N*-nitrosodimethylamine by rat liver microsomes. *Cancer Res.* (in press)
- Keighley, M.R.B., Youngs, D., Poxon, V., Morris, D., Muscroft, T.J., Burdon, D.W., Barnard, J., Bavin, P.M.G., Brimblecombe, R.W., Darkin, D.W., Moore, P.J. & Viney, N. (1984) Intra-gastric *N*-nitrosation is unlikely to be responsible for gastric carcinoma developing after operations for duodenal ulcer. *Gut*, **25**, 238-245

- Kendall, M.G. (1970) *Rank Correlation Methods*, London, Griffin
- Kershaw, N.F. & Chamberlin, N.S. (1942) Determination of nitrites. Discussion of the Shinn method as applied to examination of water. *Ind. Eng. Chem. (Anal. Ed.)*, *14*, 312-313
- Kiese, M. (1974) *Methemoglobinemia — A Comprehensive Treatise*, Cleveland, OH, CRC Press, pp. 72-73
- Kikugawa, K., Kato, T., Konoc, Y. & Sawamura, A. (1985) Effects of methyl linoleate hydroperoxide and hydrogen peroxide on the *N*-nitrosation of dimethylamine. *Food Chem. Toxicol.*, *23*, 339-342
- Kim, S., Vollberg, T.M., Ro, J.-Y., Kim, M. & Sirover, M.A. (1986) *O*<sup>6</sup>-Methylguanine methyltransferase increases before S phase in normal human cells but does not increase in hypermutable Bloom's syndrome cells. *Mutat. Res.*, *173*, 141-145
- Kim, Y.-K., Tannenbaum, S.R. & Wishnok, J.S. (1982) *Effects of ascorbic acid on the nitrosation of dialkyl amines*. In: Seib, P.A. & Tolbert, B.M., eds, *Ascorbic Acid: Chemistry, Metabolism, and Uses (Advances in Chemistry Series 200)*, Washington DC, American Chemical Society, pp. 571-585
- Klaude, M. & von der Decken, A. (1985) Methionine-cysteine deficiency and alkylation of DNA in liver, kidney and lung of mice administered dimethylnitrosamine. *Biochem. Pharmacol.*, *34*, 3627-3631
- Klaude, M. & von der Decken, A. (1986) Protein synthesis activity *in vitro* in mouse tissues in relation to methionine-cysteine deficiency and age. *Mech. Ageing Dev.*, *34*, 261-272
- Kleihues, P. & Wiestler, O.D. (1986) *Structural DNA modifications and DNA repair in organ-specific tumor induction*. In: Cohen, G.M., ed., *Target Organ Toxicity*, Vol. II, chapter 9, Boca Raton, FL, CRC Press, pp. 159-180
- Klein, C. & Klein, E. (1985) Evolution of tumours and the impact of molecular oncology. *Nature*, *315*, 190-195
- Klein, D., Gaconnet, N., Poullain, B. & Debry, G. (1978) Effect of a bolus of nitrate on salivary and gastric nitrite in man (Fr.). *Food Cosmet. Toxicol.*, *16*, 111-115
- Klein, R.G. & Schmezer, P. (1984) *Quantitative measurement of the exhalation rate of volatile N-nitrosamines in inhalation experiments with anaesthetized Sprague-Dawley rats*. In: O'Neill, I.K., von Borstel, R.C., Miller, C.T., Long, J. & Bartsch, H., eds, *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer (IARC Scientific Publications No. 57)*, Lyon, International Agency for Research on Cancer, pp. 513-517
- Kodama, M., Kodama, T., Susuki, H. & Kondo, K. (1984) Effect of rice and salty rice diet on the structure of mouse stomach. *Nutr. Cancer*, *6*, 135-147
- Koepke, S.R., Kupper, R. & Michejda, C.J. (1979) Unusually facile solvolysis of primary tosylates. A case for participation by the *N*-nitroso group. *J. org. Chem.*, *44*, 2718-2722
- Koepke, S.R., Tondeur, Y., Farrelly, J.G., Stewart, M.L., Michejda, C.J. & Kroeger-Koepke, M.B. (1984) Metabolism of <sup>15</sup>N-labelled *N*-nitrosodimethylamine and *N*-nitroso-*N*-methylaniline by isolated rat hepatocytes. *Biochem. Pharmacol.*, *33*, 1509-1513
- Koestner, A.W., Ruecker, F.A. & Koestner, A. (1977) Morphology and pathogenesis of tumors of the thymus and stomach in Sprague-Dawley rats following intragastric administration of methyl-nitrosourea (MNU). *Int. J. Cancer*, *20*, 418-426
- Kohn, K.W., Ewig, R.A.G., Erickson, L.C., Leonard, C. & Zwelling, L.A. (1981) *Measurement of strand breaks and cross-links by alkaline elution*. In: Friedberg, E.C. & Hanawalt, P.C., eds, *DNA Repair*, Vol. 1, New York, Marcel Dekker, pp. 379-401
- Kokkinakis, D.M., Hollenberg, P.F. & Scarpelli, D.G. (1985) Major urinary metabolites in hamsters and rats treated with *N*-nitroso(2-hydroxypropyl)(2-oxopropyl)amine. *Cancer Res.*, *45*, 3586-3592

- Kokkinakis, D.M., Hollenberg, P.F. & Scarpelli, D.G. (1986) The role of hepatic sulfotransferases in the activation of  $\beta$ -hydroxynitrosamines to potentially mutagenic agents. *Proc. Am. Assoc. Cancer Res.*, 27, 119
- Konishi, Y., Kondo, H., Denda, A., Takahashi, S. & Inui, S. (1978a) *Lung carcinomas induced by oral administration of N-bis(2-hydroxypropyl)nitrosamine in rats*. In: Severi, L., ed., *Tumors of Early Life in Man and Animals*, Perugia, Monteluce, pp. 637-649
- Konishi, Y., Kondo, H., Inui, S., Denda, A., Ikeda, T. & Kojima, K. (1978b) Organotropic effect of N-bis(2-hydroxypropyl)nitrosamine: production of lung and liver tumors by its oral administration in mice. *Gann*, 69, 77-84
- Konishi, Y., Iheda, T., Yoshimura, H., Kawabata, A. & Mikami, R. (1979) Carcinogenic effect of N-bis(2-hydroxypropyl) nitrosamine by a single administration in rats. *Cancer Lett.*, 6, 115-119
- Koppang, N. (1963) *Discussion contribution about etiologic changes and species occurrence, to the paper: Severe liver disease in fur-bearing animals (N. Böhler) (Norw.)*. In: Blom, Ed., ed., *Proceedings of the 9th Nordic Veterinary Congress*, Vol. II, Copenhagen, pp. 777-778
- Koppang, N. (1964) An outbreak of toxic liver injury in ruminants. *Nord. Vet.-med.*, 16, 305-322
- Koppang, N. (1966) A severe progressive liver disease in fur animals. I. Symptoms and organ changes. *Nord. Vet.-med.*, 18, 205-209
- Koppang, N. (1970) The hepatotoxic and carcinogenic actions of dimethylnitrosamine. *Acta pathol. microbiol. scand., Suppl.* 215, 30
- Koppang, N. (1974a) The toxic effects of dimethylnitrosamine in sheep. *Acta vet. scand.*, 15, 533-543
- Koppang, N. (1974b) Toxic effects of dimethylnitrosamine in cows. *J. natl. Cancer Inst.*, 52, 523-531
- Koppang, N. (1974c) Dimethylnitrosamine — formation in fish meal and toxic effects in pigs. *Am. J. Pathol.*, 74, 94-106
- Koppang, N. (1980) *A survey of feeding N-nitrosodimethylamine (NDMA) to domestic animals over an 18 year period*. In: Walker, E.A., Griciūtė, L., Castegnaro, M. & Börzsönyi, M., eds, *N-Nitroso Compounds: Analysis, Formation and Occurrence (IARC Scientific Publications No. 31)*, Lyon, International Agency for Research on Cancer, pp. 779-785
- Koppang, N. (1981) *Environmental exposure of people and animals to nitrosamines (Norw.)* In: *Det Norske videnskaps-akademis Aarbok 1980*, Oslo, Universitetsforlaget, pp. 157-168
- Koppang, N. & Helgebostad, A. (1966) Toxic hepatitis in fur animals II and III. *Nord. Vet.-med.*, 18, 210-225
- Koppang, N. & Helgebostad, A. (1987a) Dimethylnitrosamine induced toxic-, vascular and carcinogenic changes in mink (in press)
- Koppang, N. & Helgebostad, A. (1987b) Nitrosamine induced vascular and carcinogenic changes in mink (in press)
- Koppang, N. & Rimeslätten, H. (1976) *Toxic and carcinogenic effects of nitrosodimethylamine in mink*. In: Walker, E.A., Bogovski, P. & Griciūtė, L., eds, *Environmental N-Nitroso Compounds. Analysis and Formation (IARC Scientific Publications No. 14)*, Lyon, International Agency for Research on Cancer, pp. 443-452
- Koppang, N. & Slagsvold, P. (1964) Toxic hepatitis in chickens (Norw.). *Medlembst. Norsk Vet.-foren.*, 16, 251-263
- Koppang, N., Slagsvold, P., Aas Hansen, M., Sjøgnen, E. & Svenkerud, R. (1964) Feeding experiments with meal produced from herring preserved with sodium nitrite and formalin. *Nord. Vet.-med.*, 16, 343-362
- Koppang, N., Helgebostad, A., Armstrong, D. & Rimeslätten, H. (1981) Toxic and carcinogenic effects of dimethylnitrosamine (DMNA) in blue fox (*Alopex lagopus*). *Acta vet. scand.*, 22, 501-516
- Korsten, M.A., Matsuzaki, S., Feinman, L. & Lieber, C.S. (1975) High blood acetaldehyde levels after ethanol administration. *New Engl. J. Med.*, 292, 386-389

- Kramer, S., Ward, E., Meadows, A.T. & Malone, K. (1987) Medical and drug risk factors associated with neuroblastoma: a case-control study. *J. natl Cancer Inst.* (in press)
- Krauth-Siegel, R.L., Stahl, W., Schirmer, R.H. & Eisenbrand, G. (1985) Human glutathione reductase as a drug target: 2-hydroxyethylation of Cys<sub>58</sub> by 1-(2-chloroethyl)-3-(2-hydroxyethyl)-1-nitrosourea (HECNU). *Biol. Chem. Hoppe-Seyler*, *366*, 814
- Kroeger-Koepke, M.B. & Michejda, C.J. (1979) Evidence for several demethylase enzymes in the oxidation of dimethylnitrosamine and phenylmethylnitrosamine by rat liver fractions. *Cancer Res.*, *39*, 1587-1591
- Kroeger-Koepke, M.B., Koepke, S.R., McClusky, G.A., Magee, P.N. & Michejda, C.J. (1981)  $\alpha$ -Hydroxylation pathway in the in-vitro metabolism of carcinogenic nitrosamines: *N*-nitrosodimethylamine and *N*-nitroso-*N*-methylaniline. *Proc. natl Acad. Sci. USA*, *78*, 6489-6493
- Kroeger-Koepke, M.B., Reuber, M.D., Iype, P.T., Lijinsky, W. & Michejda, C.J. (1983) The effect of substituents in the aromatic ring on carcinogenicity of *N*-nitrosomethylaniline in F344 rats. *Carcinogenesis*, *4*, 157-160
- Krokan, H., Grafström, R.C., Sundqvist, K., Esterbauer, H. & Harris, C.C. (1985a) Cytotoxicity, thiol depletion and inhibition of O<sup>6</sup>-methylguanine-DNA methyltransferase by various aldehydes in cultured human bronchial fibroblasts. *Carcinogenesis*, *6*, 1755-1759
- Krokan, H., Lechner, J., Krokan, R.H. & Harris, C.C. (1985b) Normal human bronchial epithelial cells do not show an adaptive response after treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. *Mutat. Res.*, *146*, 205-209
- Krokan, H., Haugen, A., Giereksky, K.E., Nilson, I.W., Yoakum, G.H., Harris, C.C. & Myrnes, B. (1986) O<sup>6</sup>-Methylguanine-DNA methyltransferase activity in human tissues and the possible suppressive effect of some activated *ras* oncogenes in cultured cells. In: Myrnes, B. & Krokan, H., eds, *Repair of DNA Lesions Introduced by N-Nitroso Compounds*, Oslo, Norwegian University Press, pp. 199-209
- Krüger, F.W. (1971) On the metabolism of nitrosamines *in vivo*. I. Evidence for  $\beta$ -oxidation of aliphatic di-*n*-alkylnitrosamines: the simultaneous formation of 7-methylguanine as well as 7-propyl- or 7-butylguanine after application of di-*n*-propyl- or di-*n*-butylnitrosamine (Ger.). *Z. Krebsforsch.*, *76*, 147-154
- Krüger, F.W. & Bertram, B. (1973) Metabolism of nitrosamines *in vivo*. III. On the methylation of nucleic acids by aliphatic di-*n*-alkylnitrosamines *in vivo* resulting from  $\beta$ -oxidation: the formation of 7-methylguanine after application of 2-oxo-propyl-propyl-nitrosamine and methyl-propyl-nitrosamine. *Z. Krebsforsch.*, *80*, 189-196
- Kubacka, W. & Scanlan, R.A. (1984) Kinetics of nitrosation of four dipeptides. *N*-Terminal in proline. *J. Agric. Food Chem.*, *32*, 404-406
- Kubacka, W., Libbey, L.M. & Scanlan, R.A. (1984) Formation and chemical characterization of some *N*-nitrosopeptides. *N*-Terminal in proline. *J. Agric. Food Chem.*, *32*, 401-404
- Kuenzig, W., Tkaczewski, V., Kamm, J.J., Conney, A.H. & Burns, J.J. (1977) The effect of ascorbic acid deficiency on extrahepatic microsomal metabolism of drugs and carcinogens in the guinea pig. *J. Pharmacol. exp. Ther.*, *201*, 527-533
- Kunisaki, N. & Hayashi, M. (1979) Formation of *N*-nitrosamines from secondary amines and nitrite by resting cells of *Escherichia coli*. *Appl. environ. Microbiol.*, *37*, 279
- Kurechi, T. & Kikugawa, K. (1979) Nitrite-lipid reaction in aqueous system: inhibitory effects on *N*-nitrosamine formation. *J. Food Sci.*, *44*, 1263-1266, 1271
- Kurosky, A. & Hofmann, T. (1972) Kinetics of the reaction of nitrous acid with model compounds and proteins, and the conformational state of *N*-terminal groups in the chymotrypsin family. *Can. J. Biochem.*, *50*, 1282-1296
- Kuthan, H., Haussmann, H.-J. & Werringloer, J. (1984) Microsomal denitrosation of *N*-nitrosodimethylamine. In: Bors, W., Saran, M. & Tait, D., eds, *Oxygen Radicals in Chemistry and Biology*, Berlin (West), Walter de Gruyter, pp. 231-234

- Kuzmich, S., Marky, L.A. & Jones, R.A. (1983) Specifically alkylated DNA fragments. Synthesis and physical characterization of d[CGC(O<sup>6</sup>Me)GCG] and d[CGT(O<sup>6</sup>Me)GCG]. *Nucleic Acids Res.*, *11*, 3393-3404
- Kyrtopoulos, S.A. & Swann, P.F. (1980) The use of radioimmunoassay to study the formation and excision of O<sup>6</sup>-methylguanine in mouse liver main-band and satellite DNA following dimethylnitrosamine administration. *J. Cancer Res.*, *98*, 127-131
- Kyrtopoulos, S.A., Daskalakis, G., Legakis, N.I., Konidaris, N., Psarrou, E., Bonatsos, G., Golematis, B., Lakiotis, G., Bliouras, N. & Outram, J.R. (1985a) Studies in gastric carcinogenesis. II. Absence of elevated concentrations of N-nitroso compounds in the gastric juice of Greek hypochlorhydric individuals. *Carcinogenesis*, *6*, 1135-1140
- Kyrtopoulos, S.A., Daskalakis, G. & Outram, J.R. (1985b) Studies in gastric carcinogenesis. III. The kinetics of nitrosation of gastric-juice components *in vitro* and their implications for the *in vivo* formation of N-nitroso compounds in normal and in hypochlorhydric populations. *Carcinogenesis*, *6*, 1141-1145
- Labuc, G.E. & Archer, M.C. (1982) Esophageal and hepatic microsomal metabolism of N'-nitrosomethylbenzylamine and N-nitrosodimethylamine in the rat. *Cancer Res.*, *42*, 3181-3186
- Ladd, K.F., Archer, M.C. & Newmark, H.L. (1984a) *Increased endogenous nitrosation in smokers*. In: O'Neill, I.K., von Borstel, R.C., Miller, C.T., Long, J. & Bartsch, H., eds, *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer (IARC Scientific Publications No. 57)*, Lyon, International Agency for Research on Cancer, pp. 811-817
- Ladd, K.F., Newmark, H.L. & Archer, M.C. (1984b) N-Nitrosation of proline in smokers and nonsmokers. *J. natl Cancer Inst.*, *73*, 83-87
- Lai, D.Y., Arcos, J.C. & Argus, M.F. (1979a) Factors influencing the microsome- and mitochondria-catalyzed *in-vitro* binding of diethylnitrosamine and N-nitrosopiperidine to deoxyribonucleic acid. *Biochem. Pharmacol.*, *28*, 3545-3550
- Lai, D.Y., Myers, S.C., Woo, Y.-T., Greene, E.J., Friedman, M.A., Argus, M.F. & Arcos, J.C. (1979b) Role of dimethylnitrosamine-demethylase in the metabolic activation of dimethylnitrosamines. *Chem.-biol. Interactions*, *28*, 107-126
- Lake, B.G., Collins, M.A., Harris, R.A., Phillips, J.C., Cottrell, R.C. & Gangolli, S.D. (1982a) Studies on the metabolism of dimethylnitrosamine *in vitro* by rat-liver preparations. I. Comparison with mixed function oxidase enzymes. *Xenobiotica*, *12*, 435-445
- Lake, B.G., Harris, R.A., Collins, M.A., Cottrell, R.C., Phillips, J.C. & Gangolli, S.D. (1982b) Studies on the metabolism of dimethylnitrosamine *in vitro* by rat liver preparations. II. Inhibition by substrates and inhibitors of monoamine oxidase. *Xenobiotica*, *12*, 567-579
- Lane, R.P. & Bailey, M.E. (1973) The effect of pH on dimethylnitrosamine formation in human gastric cancer. *Food Cosmet. Toxicol.*, *11*, 851-854
- Langenbach, R., Gingell, R., Kuszynski, C., Walker, B., Nagel, D. & Pour, P. (1980) Mutagenic activities of oxidized derivatives of N-nitrosodipropylamine in the liver cell-mediated and *Salmonella typhimurium* assays. *Cancer Res.*, *40*, 3463-3467
- Langenbach, R., Nesnow, S. & Rice, J.M., eds (1983) *Organ and Species Specificity in Chemical Carcinogenesis*, New York, Plenum
- Lans, M., de Gerlache, J., Taper, H., Pr at, V. & Roberfroid, M. (1983) Phenobarbital as a promoter in the initiation/selection process of experimental rat hepatocarcinogenesis. *Carcinogenesis*, *4*, 141-144
- Lau, S.S., McMahon, J.B., McMenamin, M.G., Schuller, H.M. & Boyd, M.R. (1986) Differential metabolism of arachidonic acid in human lung cancer cell lines. *Cancer Res.* (in press)
- Lawley, P.D. (1984) *Carcinogenesis by alkylating agents*. In: Searle, C.E., ed., *Chemical Carcinogens (ACS Symposium Series 182)* Washington DC, American Chemical Society, pp. 325-383
- Lawson, T.A., Gingell, R., Nagel, D., Hines, L. & Ross, A. (1981) Methylation of hamster DNA by the carcinogen N-nitrosobis(2-oxopropyl)amine. *Cancer Lett.*, *11*, 251-254



- Leach, S.A., Challis, B.C., Cook, A.R., Hill, M.J. & Thompson, M.H. (1985a) The catalysis of nitrosamine formation by clinical isolates of bacteria. *Br. J. Cancer*, *51*, 585
- Leach, S.A., Challis, B.C., Cook, A.R., Hill, M.J. & Thompson, M.H. (1985b) Bacterial catalysis of the *N*-nitrosation of secondary amines. *Biochem. Soc. Trans.*, *13*, 381-382
- Leach, S.A., Challis, B.C., Cook, A.R., Hill, M.J. & Thompson, M.H. (1986a) Some preliminary evaluations of bacterial *N*-nitrosation reactions. *Br. J. Cancer*, *30* (Suppl.), S27-S28
- Leach, S.A., Challis, B.C., Cook, A.R., Hill, M.J. & Thompson, M.H. (1986b) A model describing the potential role of bacteria and *N*-nitroso compounds in gastric carcinogenesis. *Cancer Lett.* (in press)
- Leaver, D.D., Swann, P.F. & Magee, P.N. (1969) The induction of tumours in the rat by a single oral dose of *N*-nitrosomethylurea. *Br. J. Cancer*, *23*, 177-187
- Lechner, J.F., Stoner, G.D., Yoakum, G.H., Willey, J.C., Gräfstrom, R.C., Masui, T., LaVeck, M.A. & Harris, C.C. (1986) *In vitro* carcinogenesis studies with human tracheobronchial tissues and cells. In: Schiff, L.J., ed., *In Vitro Models of Respiratory Epithelium*, Boca Raton, FL, CRC Press, pp. 143-159
- Lee, S. Y. & Guttenplan, J.B. (1981) A correlation between mutagenic and carcinogenic potencies in a diverse group of *N*-nitrosamines: determination of mutagenic activities of weakly mutagenic *N*-nitrosamines. *Carcinogenesis*, *2*, 1339-1344
- Lesca, P. (1984) Protective effects of ellagic acid and other plant phenols on benzo[*a*]pyrene-induced neoplasia in mice. *Carcinogenesis*, *4*, 1651-1653
- Li, C.C., Yu, M.C. & Henderson, B.E. (1985) Some epidemiologic observations of nasopharyngeal carcinoma in Guangdong, People's Republic of China. *Natl Cancer Inst. Monogr.*, *69*, 49-52
- Li, M., Cheng, S., Tian, G., Lu, S. & Zhao, X. (1985) Experimental studies on the induction of esophageal cancer in chickens by methylbenzyl nitrosamine. *Acta zool. sin.*, *31*, 34-38
- Li, M.H., Lu, S.H., Ji, C., Cheng, S.J., Wang, M.Y., Jin, C.L. & Wang, Y.L. (1978) *Experimental studies on the formation of nitrosamines in vivo and in vitro* (Chin.). In: *Collection of Papers for the Twentieth Anniversary of the Cancer Institute and Ritan Hospital*, Beijing, pp. 75-78
- Li, M.H., Lu, S.H., Ji, C., Wang, M.Y., Cheng, S. & Jin, C. (1979) Formation of carcinogenic *N*-nitroso compounds in corn-bread inoculated with fungi. *Sci. Sin.*, *22*, 471-477
- Lijinsky, W. (1974) Reaction of drugs with nitrous acid as source of carcinogenic nitrosamines. *Cancer Res.*, *34*, 255-258
- Lijinsky, W. (1984a) Species differences in nitrosamine carcinogenesis. *J. Cancer Res. clin. Oncol.*, *108*, 46-55
- Lijinsky, W. (1984b) *Structure-activity relation in carcinogenesis by N-nitroso compounds*. In: Rao, T.K., Lijinsky, W. & Epler, J.L., eds, *Genotoxicology of N-Nitroso Compounds*, New York, Plenum Press, pp. 189-231
- Lijinsky, W. (1985) the metabolism and cellular interactions of some aliphatic nitrogenous carcinogens. *Cancer Lett.*, *26*, 33-42
- Lijinsky, W. & Andrews, A.W. (1983) the superiority of hamster liver microsomal fraction for activating nitrosamines to mutagens in *Salmonella typhimurium*. *Mutat. Res.*, *111*, 135-144
- Lijinsky, W. & Kovatch, R.M. (1985) Induction of liver tumors in rats by nitrosodiethanolamine at low doses. *Carcinogenesis*, *6*, 1679-1681
- Lijinsky, W. & Reuber, M.D. (1980) Carcinogenicity in rats of nitrosomethylethylamines labelled with deuterium in several positions. *Cancer Res.*, *40*, 19-21
- Lijinsky, W. & Reuber, M.D. (1983) Carcinogenicity of hydroxylated alkyl nitrosoureas and of nitrosooxazolidones by mouse skin painting and by gavage in rats. *Cancer Res.*, *43*, 214-221
- Lijinsky, W. & Reuber, M.D. (1984) Dose-response study with *N*-nitrosodiethanolamine in F344 rats. *Food Chem. Toxicol.*, *22*, 23-26

- Lijinsky, W. & Ross, A.E. (1969) Alkylation of rat liver nucleic acids not related to carcinogenesis by *N*-nitrosamines. *J. natl Cancer Inst.*, *42*, 1095-1100
- Lijinsky, W. & Taylor, H.W. (1975a) Tumorigenesis by oxygenated nitrosopiperidine in rats. *J. natl Cancer Inst.*, *55*, 705-707
- Lijinsky, W. & Taylor, H.W. (1975b) Increased carcinogenicity of 2,6-dimethylnitrosomorpholine compared with nitrosomorpholine in rats. *Cancer Res.*, *35*, 2123-2125
- Lijinsky, W. & Taylor, H.W. (1978) Relative carcinogenic effectiveness of derivatives of nitrosodiethylamine in rats. *Cancer Res.*, *38*, 2391-2394
- Lijinsky, W., Loo, J. & Ross, A.E. (1968) Mechanism of alkylation of nucleic acids by nitrosodimethylamine. *Nature*, *218*, 1174-1175
- Lijinsky, W., Keefer, L. & Loo, J. (1970) The preparation and properties of some nitrosamino acids. *Tetrahedron*, *26*, 5137-5153
- Lijinsky, W., Keefer, L., Conrad, E. & Van de Bogart, R. (1972) Nitrosation of tertiary amines and some biologic implications. *J. natl Cancer Inst.*, *49*, 1239-1249
- Lijinsky, W., Keefer, L., Loo, J. & Ross, A.E. (1973) Studies of methylation of nucleic acids in rats by cyclic nitrosamines. *Cancer Res.*, *33*, 1634-1641
- Lijinsky, W., Reuber, M.D. & Manning, W.B. (1980) Potent carcinogenicity of nitrosodiethanolamine in rats. *Nature*, *288*, 589-590
- Lijinsky, W., Saavedra, J.E. & Reuber, M.D. (1981) Induction of carcinogenesis in Fischer rats by methylalkylnitrosamines. *Cancer Res.*, *41*, 1288-1292
- Lijinsky, W., Singer, G.M. & Kovatch, R.M. (1985) Similar carcinogenic effects in rats of 1-ethyl-1-nitroso-3-hydroxyethylurea and 1-hydroxyethyl-1-nitroso-3-ethylurea. *Carcinogenesis*, *6*, 641-643
- Likhachev, A. (1985) *Effect of age on DNA repair in carcinogenesis due to alkylating agents*. In: Likhachev, A., Anisimov, V. & Montesano, R., eds, *Age-related Factors in Carcinogenesis (IARC Scientific Publications No. 58)*, Lyon, International Agency for Research on Cancer, pp. 239-246
- Little, J.B. & Kennedy, A.R. (1979) Evaluation of alpha radiation-induced respiratory carcinogenesis in Syrian hamsters: total dose and dose-rate. *Prog. exp. Tumor Res.*, *24*, 356-369
- Litvinov, N.N., Voronin, V.M. & Kazachkov, V.I. (1986) Studies of carcinogenesis modifying properties of aniline, carbon tetrachloride, benzene and ethanol (Russ.). *Eksp. Onkol.*, *8*, 21-23
- Loeppky, R.N., Tomasik, W., Kovacs, D.A., Outram, J.R. & Byington, K.H. (1984a) *Alternative bioactivation routes for  $\beta$ -hydroxynitrosamines. Biochemical and chemical model studies*. In: O'Neill, I.K., von Borstel, R.C., Miller, C.T., Long, J. & Bartsch, H., eds, *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer (IARC Scientific Publications No. 57)*, Lyon, International Agency for Research on Cancer, pp. 429-436
- Loeppky, R.N., Tomasik, W. & Millard, T.G. (1984b) *Ester-mediated nitrosamine formation from nitrite and secondary or tertiary amines*. In: O'Neill, I.K., von Borstel, R.C., Miller, C.T., Long, J. & Bartsch, H., eds, *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer (IARC Scientific Publications No. 57)*, Lyon, International Agency for Research on Cancer, pp. 353-363
- Löfberg, B. & Tjälve, H. (1984) The disposition and metabolism of *N*-nitrosodiethylamine in adult, infant and foetal tissues of the Syrian golden hamster. *Acta pharmacol. toxicol.*, *54*, 104-114
- Löfberg, B. & Tjälve, H. (1985) Tissue specificity of *N*-nitrosomorpholine metabolism in Sprague-Dawley rats. *Food Chem. Toxicol.*, *23*, 647-654
- Long, C.L., King, E.J. & Sperry, W.M. (1961) In: *Biochemists' Handbook*, London, E. & F.N. Spon, Ltd, p. 911
- Looker, J.H. & Carpenter, J.W. (1967) *N*-(2-Diazo-3-oxoalkanoyl)-glycine esters. I. Synthesis of 4- and 5-phenoxy derivatives and isomerization of *N*-(2-diazo-3-oxo-3-phenylpropanoyl) glycine ethyl ester. *Can. J. Chem.*, *45*, 1727-1734

- Lorr, N.A., Tu, Y.Y. & Yang, C.S. (1982) The nature of nitrosamine denitrosation by rat liver microsomes. *Carcinogenesis*, *3*, 1039-1043
- Lotlikar, P.D., Insetta, S., Lyons, P.R. & Jhee, E.C. (1980) Inhibition of microsome mediated binding of aflatoxin B<sub>1</sub> to DNA by glutathione S-transferases. *Cancer Lett.*, *9*, 143-149
- Lotlikar, P.D., Jhee, E.C., Insetta, S.M. & Clearfield, M.S. (1984) Modulation of microsome mediated aflatoxin B<sub>1</sub> binding to exogenous and endogenous DNA by cytosolic glutathione S-transferases in rat and hamster livers. *Carcinogenesis*, *5*, 269-276
- Lown, J.W. & McLaughlin, L.W. (1979) Nitrosourea-induced DNA single-strand breaks. *Chem. Pharmacol.*, *28*, 1631-1638
- Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J. biol. Chem.*, *193*, 265-275
- Lu, S.H. (1977) Preliminary investigation on the carcinogenicity of extracts of pickled vegetables in Linxian. *Res. Cancer Prev. Treat.*, *2*, 46-49
- Lu, S.H. (1982) Screening for cancer of the oesophagus in northern China (Fr.). *Quest. Med.*, *35*, 1193-1195
- Lu, S.H. & Cheng, S.J. (1978) Determination of the nitrite contents in the saliva and gastric juice of the population in Linxian. *Res. Cancer Prev. Treat.*, *1*, 17-20
- Lu, S.H. & Lin, P. (1982) Recent research on the etiology of esophageal cancer in China. *Z. Gastroenterol.*, *20*, 361-367
- Lu, S.H., Ji, C., Jin, C.L., Wu, L.L. & Chui, L.X. (1978) *Analysis of nitrosamines in foods collected from Linxian and Fanxian* (Chin.). In: *Collection of Papers for the Twentieth Anniversary of the Cancer Institute and Ritan Hospital*, Beijing, pp. 38-45
- Lu, S.H., Camus, A.-M., Tomatis, L. & Bartsch, H. (1981) Mutagenicity of extracts of pickled vegetables collected in Linhsian county, a high incidence area for esophageal cancer in Northern China. *J. natl Cancer Inst.*, *66*, 33-36
- Lu, S.-H., Ohshima, H., Fu, H.-M., Tian, Y., Li, F.-M., Blettner, M., Wahrendorf, J. & Bartsch, H. (1986) Urinary excretion of *N*-nitrosamino acids and nitrate by inhabitants of high- and low-risk areas for esophageal cancer in northern China: endogenous formation of nitrosoproline and its inhibition by vitamin C. *Cancer Res.*, *46*, 1485-1491
- Lukhovin, G.M., Pshezhetsky, V.S. & Muntazaev, G.A. (1973) *Eur. Poly. J.*, *9*, 559-565
- Lynch, S.C., Gruenwedel, D.W. & Russel, G.F. (1983) Mutagenic activity of a nitrosated early Maillard product: DNA synthesis (DNA repair) induced in HeLa S3 carcinoma cells by nitrosated 1(*N*-L-tryptophan)-1-deoxy-D-fructose. *Food Chem. Toxicol.*, *21*, 551-556
- MacKenzie, W.F. & Garner, F.M. (1973) Comparison of neoplasms in six sources of rats. *J. natl Cancer Inst.*, *50*, 1243-1257
- Magee, P.N. (1956) Toxic liver injury. The metabolism of dimethylnitrosamine. *Biochem. J.*, *64*, 676-682
- Magee, P.N. (1980) *Metabolism of nitrosamines: an overview*. In: Coon, M.J., Conney, A.H., Estabrook, R.W., Gelboin, H.V., Gillette, J.R. & O'Brien, P.J., eds, *Microsomes, Drug Oxidations, and Chemical Carcinogenesis*, Vol. II, New York, Academic Press, pp. 1081-1092
- Magee, P.N., ed. (1982) *Nitrosamines and Human Cancer (Banbury Report 12)*, Cold Spring Harbor, NY, CSH Press
- Magee, P.N. & Barnes, J.M. (1967) Carcinogenic *N*-nitroso compounds. *Adv. Cancer Res.*, *10*, 164-246
- Magee, P.N., Montesano, R. & Preussmann, R. (1976) *N-Nitroso compounds and related carcinogens*. In: Searle, C.E., ed., *Chemical Carcinogens (ACS Symposium Series 173)*, Washington DC, American Chemical Society, pp. 491-625
- Malaveille, C., Brun, G., Kolar, G. & Bartsch, H. (1982) Mutagenic and alkylating activities of 3-methyl-1-phenyl triazenes and their possible role as carcinogenic metabolites of the parent dimethyl compounds. *Cancer Res.*, *42*, 1446-1453

- Male, R., Nes, I.F. & Kleppe, K. (1981) Purification and properties of 3-methyladenine DNA glycosylase from L-cells. *Eur. J. Biochem.*, *121*, 243-249
- Mandel, L.R., Srinivasan, P.R. & Borek, E. (1966) Origin of urinary methylated purines. *Nature*, *209*, 586-588
- Maniatis, T., Fritsch, E.F. & Sambrook, J., eds (1982) *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor, NY, CSH Press
- Manning, M.D. & Carroll, B.E. (1957) Some epidemiological aspects of leukemia in children. *J. natl Cancer Inst.*, *19*, 1087-1094
- Mantel, N. & Haenzel, W. (1959) Statistical aspects of the analysis of data from retrospective studies of disease. *J. natl Cancer Inst.*, *22*, 719-748
- Marasas, W.F.O., Wehner, F.C., van Rensburg, S.J. & van Schalkwyk, D.J. (1981) Mycoflora of corn produced in human esophageal cancer areas in Transkei, Southern Africa. *Phytopathology*, *71*, 792-796
- Marasas, W.F.O., Krick, N.P.J., Fincham, J.E. & van Rensburg, S.J. (1984) Primary liver cancer and esophageal basal cell hyperplasia in rats caused by *Fusarium moniliforme*. *Int. J. Cancer*, *34*, 383-387
- Margison, G.P. (1985) *The effects of age on the metabolism of chemical carcinogens and inducibility of O<sup>6</sup>-methylguanine methyltransferase*. In: Likhachev, A., Anisimov, V. & Montesano, R., eds, *Age-related Factors in Carcinogenesis (IARC Scientific Publications No. 58)*, Lyon, International Agency for Research on Cancer, pp. 225-237
- Maron, D. & Ames, B.N. (1983) Revised methods for the *Salmonella* mutagenicity test. *Mutat. Res.*, *113*, 173-215
- Massey, R.C., Crews, C. & McWeeny, D.J. (1982) Method for high-performance liquid chromatographic measurement of *N*-nitrosamines in food and beverages. *J. Chromatogr.*, *241*, 423-427
- Massey, R.C., Key, P.E., McWeeny, D.J. & Knowles, M.E. (1984a) The application of a chemical denitrosation and chemiluminescence detection procedure for estimation of the apparent concentration of total *N*-nitroso compounds in foods and beverages. *Food Addit. Contam.*, *1*, 11-16
- Massey, R.C., Bayly, J.M., Key, P.E., McWeeny, D.J. & Knowles, M.E. (1984b) Total *N*-nitroso group analysis of foods. II. Further studies on the precision and sensitivity of the assay. *Food Addit. Contam.*, *1*, 237-244
- Masson, H.A., Ioanides, C. & Gibson, G.G. (1983) The role of highly purified forms of rat liver cytochrome P-450 in the demethylation of dimethylnitrosamine and its activation to mutagens. *Toxicol. Lett.*, *17*, 131-135
- Matsuya, T. (1982) Alcohol intake and smoking habits of Japanese telephone employees and their effects on dietary habits. *J. Formosan med. Assoc.*, *81*, 857-867
- McCarthy, T.V., Karran, P. & Lindahl, T. (1984) Inducible repair of *O*-alkylated pyrimidines in *E. coli*. *EMBO J.*, *3*, 545-550
- McCoy, G.D., Katayama, S., Young, R., Wyatt, M. & Hecht, S. (1982) *Influence of chronic ethanol consumption on the metabolism and carcinogenicity of tobacco-related carcinogens*. In: Bartsch, H., O'Neill, I.K., Castegnaro, M. & Okada, M., eds, *N-Nitroso Compounds. Occurrence and Biological Effects (IARC Scientific Publications No. 41)*, Lyon, International Agency for Research on Cancer, pp. 635-642
- McGregor, C., Schnaitman, C., Normansell, D. & Hodgins, M. (1974) Purification and properties of nitrate-reductase from *Escherichia coli* K12. *J. biol. Chem.*, *249*, 5321-5327
- McLaughlin, P.J. & Weihrauch, J.L. (1979) Vitamin E content of foods. *J. Am. Diet Assoc.*, *75*, 647-665

- Mehta, F.S., Pindborg, J.J., Hammer, J.E., Gupta, P.C., Daftary, D.K., Sahiar, B.E., Shroff, B.C., Sanghvi, L.D., Bhonsle, R.B., Choksi, S.K., Dandekar, V.V., Mehta, Y.N., Pitkar, V.K., Sinor, P.N., Shah, N.C., Turner, P.S. & Upadhyay, S.A. (1971) *Report on Investigations of Oral Cancer and Precancerous Conditions in Indian Rural Populations, 1966-1969*, Copenhagen, Munksgaard, pp. 48, 68, 89, 107, 120
- Meinsma, L. (1964) Nutrition and cancer (Dutch) *Voeding*, 25, 357-365
- Melzak, J. (1966) The incidence of bladder cancer in paraplegia. *Paraplegia*, 4, 85-96
- Menkveld, G.J., Van Der Laken, C.J., Hermsen, T., Kriek, E., Scherer, E. & Den Engelse, L. (1985) Immunohistochemical localization of O<sup>6</sup>-ethyldeoxyguanosine and deoxyguanosin-8-yl(acetyl)-aminofluorene in liver sections of rats treated with diethylnitrosamine, ethylnitrosourea or N-acetylaminofluorene. *Carcinogenesis*, 6, 263-270
- Meselson, M. & Russell, K. (1977) *Comparisons of carcinogenic and mutagenic potency*. In: Hiatt, H.H., Watson, J.D. & Winsten, J.A., eds, *Origins of Human Cancer, Book C, Human Risk Assessment*, Cold Spring Harbor, NY, CSH Press, pp. 1473-1481
- Mester, L. & Mester, M. (1975) 1-Desoxy-1-(5-hydroxytryptamino)-D-fructose: a new reducing sugar derivative of serotonin. *J. Carbohydr. Nucleosides Nucleotides*, 2, 141-146
- Meyer, D.J. (1973) Interaction of cytochrome oxidases a<sub>3</sub>, and d with nitrite. *Nature-New Biol.*, 245, 276-277
- Michejda, C.J. (1986) *Annual Report 1986, Basic Science Program*, National Cancer Institute, Frederick Cancer Research Facility, Frederick, MD, p. 106
- Michejda, C.J., Andrews, A.W. & Koepke, S.R. (1979) Derivatives of side-chain hydroxylated nitrosamines direct acting mutagens in *Salmonella typhimurium*. *Mutat. Res.*, 67, 301-308
- Michejda, C.J., Kroeger-Koepke, M.B., Koepke, S.R. & Sieh, D.H. (1981) *Activation of nitrosamines to biological alkylating agents*. In: Scanlan, R.A. & Tannenbaum, S.R., eds, *N-Nitroso Compounds (ACS Symposium Series 174)*, Washington DC, American Chemical Society, pp. 3-20
- Michejda, C.J., Kroeger-Koepke, M.B., Koepke, S.R., Magee, P.N. & Chu, C. (1982) *Nitrogen formation during in vivo and in vitro metabolism of N-nitrosamines*. In: Magee, P.N., ed., *Nitrosamines and Human Cancer (Banbury Report 12)*, Cold Spring Harbor, NY, CSH Press, pp. 69-85
- Mico, B.A., Swagzdis, J.E., Hu, H.S.-W., Keefer, L.K., Oldfield, N.F. & Garland, W.A. (1985) Low dose in vivo pharmacokinetics and deuterium isotope effect studies of N-nitrosodimethylamine in rats. *Cancer Res.*, 45, 6280-6285
- Miller, J.A. (1970) Carcinogenesis by chemicals. *Cancer Res.*, 30, 559-576
- Miller, J.A. & Miller, E.C. (1977) *Ultimate chemical carcinogens as reactive mutagenic electrophiles*. In: Hiatt, H.H., Watson, J.D. & Winsten, J.A., eds, *Origins of Human Cancer*, Cold Spring Harbor, NY, CSH Press, pp. 605-627
- Milo, G. & DiPaolo, J. (1978) Neoplastic transformation of human diploid cells *in vitro* after chemical carcinogen treatment. *Nature*, 275, 130-132
- Milstein, S. & Guttenplan, J.B. (1979) Near quantitative production of molecular nitrogen from metabolism of dimethylnitrosamine. *Biochem. biophys. Res. Commun.*, 87, 337-342
- Milton-Thompson, G.J., Ahmet, Z., Barnard, J., Brimblecombe, R.W., Moore, P.J., Lightfoot, N.F., Hunt, R.H., Bavin, P.M., Darkin, D.W. & Viney, N. (1982) Intragastric acidity, bacteria, nitrite and N-nitroso compounds before, during and after cimetidine treatment. *Lancet*, i, 1091-1095
- Mirvish, S.S. (1972) *Kinetics of N-nitrosation reaction in relation to tumorigenesis experiments with nitrite plus amines or ureas*. In: Bogovski, P., Preussmann, R. & Walker, E.A., eds, *N-Nitroso Compounds: Analysis and Formation (IARC Scientific Publications No. 3)*, Lyon, International Agency for Research on Cancer, pp. 104-108
- Mirvish, S.S. (1975) Formation of N-nitroso compounds: chemistry, kinetics, and in vivo occurrence. *Toxicol. appl. Pharmacol.*, 31, 325-351

- Mirvish, S.S. (1977) *N*-Nitroso compounds: their chemical and in vivo formation and possible importance as environmental carcinogens. *J. Toxicol. environ. Health*, 2, 1267-1277
- Mirvish, S.S. (1983) The etiology of gastric cancer. Intra-gastric nitrosamide formation and other theories. *J. natl Cancer Inst.*, 71, 629-647
- Mirvish, S.S. (1985) Gastric cancer and salivary nitrate and nitrite. *Nature*, 315, 461-462
- Mirvish, S.S. & Sams, J.P. (1984) A nitrosating agent from the reaction of atmospheric nitrogen dioxide ( $\text{NO}_2$ ) with methyl linoleate: comparison with a product from the skins of  $\text{NO}_2$ -exposed mice. In: O'Neill, I.K., von Borstel, R.C., Miller, C.T., Long, J. & Bartsch, H., eds, *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer (IARC Scientific Publications No. 57)*, Lyon, International Agency for Research on Cancer, pp. 283-289
- Mirvish, S.S., Wallcave, L., Eagen, M. & Shubik, P. (1972) Ascorbate-nitrite reaction: possible means of blocking the formation of carcinogenic *N*-nitroso compounds. *Science*, 177, 65-68
- Mirvish, S.S., Cardesa, A., Wallcave, L. & Shubik, P. (1973a) Effect of sodium ascorbate on lung adenoma induction by amines plus nitrite. *Proc. Am. Assoc. Cancer Res.*, 14, 102
- Mirvish, S.S., Sams, J., Fan, T.Y. & Tannenbaum, S.R. (1973b) Kinetics of nitrosation of the amino acids proline, hydroxyproline, and sarcosine. *J. natl Cancer Inst.*, 51, 1833-1839
- Mirvish, S.S., Cardesa, A., Wallcave, L. & Shubik, P. (1975) Induction of mouse lung adenomas by amines or ureas plus nitrite and by *N*-nitroso compounds: effect of ascorbate, gallic acid, thiocyanate, and caffeine. *J. natl Cancer Inst.*, 55, 633-636
- Mirvish, S.S., Bulay, O., Runge, R.G. & Patil, K. (1980a) Study of the carcinogenicity of large doses of dimethylnitramine, *N*-nitroso-L-proline, and sodium nitrite administered in drinking water to rat. *J. natl Cancer Inst.*, 64, 1435-1442
- Mirvish, S.S., Karlowski, K., Birt, F. & Sams, J.P. (1980b) Dietary and other factors affecting nitrosomethylurea (NMU) formation in the rat stomach. In: Walker, E.A., Castegnaro, M., Gričič, L. & Börzsönyi, M., eds, *N-Nitroso Compounds: Analysis, Formation and Occurrence (IARC Scientific Publications No. 31)*, Lyon, International Agency for Research on Cancer, pp. 271-277
- Mirvish, S.S., Issenberg, P. & Sams, J.P. (1981) A study of *N*-nitrosomorpholine synthesis in rodents exposed to nitrogen dioxide and morpholine. In: Scanlan, R.A. & Tannenbaum, S.R., eds, *N-Nitroso Compounds (ACS Symposium Series 174)*, Washington DC, American Chemical Society, pp. 181-191
- Mirvish, S.S., Sams, J.P. & Issenberg, P. (1983) The nitrosating agent in mice exposed to nitrogen dioxide: improved extraction method and localization in the skin. *Cancer Res.*, 43, 2550-2554
- Mirvish, S.S., Wang, M.-Y., Smith, J.W., Deshpande, A.D., Makary, M.H. & Issenberg, P. (1985)  $\beta$ -to  $\omega$ -Hydroxylation of the esophageal carcinogen methyl-*n*-amyl nitrosamine by the rat esophagus and related tissues. *Cancer Res.*, 45, 577-583
- Mirvish, S.S., Babcock, D.M., Deshpande, A.D. & Nagel, D.L. (1986) Identification of cholesterol as a mouse skin lipid that reacts with nitrogen dioxide to yield a nitrosating agent (NSA), and of cholesteryl nitrite as the NSA produced in a chemical system from cholesterol. *Cancer Lett.*, 31, 97-104
- Mochizuki, M., Suzuki, E., Anjo, T., Wakabayashi, Y. & Okada, M. (1979) Mutagenic and DNA-damaging effects of *N*-alkyl-*N*-( $\alpha$ -acetoxyalkyl)nitrosamines, models for metabolically activated *N,N*-dialkyl nitrosamines. *Gann*, 70, 663-670
- Mochizuki, M., Anjo, T. & Okada, M. (1980a) Isolation and characterization of *N*-alkyl-*N*-(hydroxymethyl)nitrosamines from *N*-alkyl-*N*-(hydroperoxymethyl)nitrosamines by deoxygenation. *Tetrahedron Lett.*, 21, 3693-3696
- Mochizuki, M., Anjo, T., Wakabayashi, Y., Sone, Y. & Okada, M. (1980b) Formation of *N*-alkyl-*N*-(1-hydroperoxyalkyl)nitrosamines from *N*-alkyl-*N*-(1-acetoxyalkyl)nitrosamines. *Tetrahedron Lett.*, 21, 1761-1764

- Mochizuki, M., Anjo, T., Takeda, K., Suzuki, E., Sekiguchi, N., Huang, G.-F. & Okada, M. (1982) *Chemistry and mutagenicity of  $\alpha$ -hydroxy nitrosamines*. In: Bartsch, H., O'Neill, I.K., Castegnaro, M. & Okada, M., eds, *N-Nitroso Compounds: Occurrence and Biological Effects (IARC Scientific Publications No. 41)*, Lyon, International Agency for Research on Cancer, pp. 553-559
- Modan, B., Lubin, F., Barel, V., Greenberg, R.A., Modan, M. & Graham, S. (1974) The role of starches in the etiology of gastric cancer. *Cancer*, *34*, 2087-2092
- Montesano, R. & Magee, P.N. (1974) *Comparative metabolism in vitro of nitrosamines in various animal species including man*. In: Montesano, R. & Tomatis, L., eds, *Chemical Carcinogenesis Essays (IARC Scientific Publications No. 10)*, Lyon, International Agency for Research on Cancer, pp. 39-56
- Montesano, R., Becker, R., Hall, J., Likhachev, A., Lu, S.H., Umbenhauer, D. & Wild, C.P. (1985) Repair of DNA alkylation adducts in mammalian cells. *Biochimie*, *67*, 919-928
- Monti-Bragadin, C., Tamaro, M. & Banfi, E. (1974) Mutagenic activity of diazoacetyl glycine derivatives. *Antimicrob. Agents Chemother.*, *6*, 655-656
- Moore, M.A. & Kitagawa, T. (1986) Hepatocarcinogenesis in the rat: the effect of promoters and carcinogens in vivo and in vitro. *Int. Rev. Cytol.*, *101*, 125-173
- Morgan, R.W., Jain, M., Miller, A.B., Choi, N.W., Matthews, V., Munan, L., Burch, J.D., Feather, J., Howe, G.R. & Kelly, A. (1978) A comparison of dietary methods in epidemiologic studies. *Am. J. Epidemiol.*, *107*, 488-498
- Mori, Y., Takahashi, H., Yamazaki, H., Toyoshi, K., Makino, T., Yokose, Y. & Konishi, Y. (1984) Distribution, metabolism and excretion of *N*-nitrosobis(2-hydroxypropyl)amine in Wistar rats. *Carcinogenesis*, *5*, 1443-1447
- Mori, Y., Yamazaki, H., Toyoshi, K., Emi, Y., Uchida, K., Tsutsumi, M. & Konishi, Y. (1985) Inhibitory effect of organic solvents on the mutagenicity of *N*-nitrosodialkylamines in *Salmonella*. *Mutat. Res.*, *142*, 153-158
- Mori, Y., Yamazaki, H., Toyoshi, K., Denda, A. & Konishi, Y. (1986a) Mutagenic activation of carcinogenic *N*-nitrosopropylamines by liver S9 fractions from mice, rats and hamsters: evidence for a cytochrome P-450 dependent reaction. *Carcinogenesis*, *7*, 375-379
- Mori, Y., Yamazaki, H., Toyoshi, K., Maruyama, H. & Konishi, Y. (1986b) Activation of carcinogenic *N*-nitrosopropylamines to mutagens by lung and pancreas S9 fractions from various animal species and man. *Mutat. Res.*, *160*, 159-169
- Morimoto, K., Tanaka, A. & Yamaha, T. (1983) Reaction of 1-*n*-propyl-1-nitrosourea with DNA *in vitro*. *Carcinogenesis*, *4*, 1455-1458
- Morimoto, K., Dolan, M.E., Scicchitano, D. & Pegg, A.E. (1985) Repair of *O*<sup>6</sup>-propylguanine and *O*<sup>6</sup>-butylguanine in DNA by *O*<sup>6</sup>-alkylguanine-DNA-alkyltransferases from rat liver and *E. coli*. *Carcinogenesis*, *6*, 1027-1031
- Morishita, Y. & Shimizu, T. (1983) Promoting effect of intestinal *Pseudomonas aeruginosa* on gastric tumorigenesis in rats with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. *Cancer Lett.*, *77*, 347-352
- Morson, B.C., Sobin, L.H., Johansen, A., Nagayo, T. & Serck-Hanssen, A. (1980) Precancerous conditions and epithelial dysplasia in the stomach. *J. clin. Pathol.*, *33*, 711-721
- Mueller, R.L., Hagel, H.-J., Greim, G., Rupp, H. & Domschke, W. (1983) Endogenous synthesis of carcinogenic *N*-nitroso compounds: bacterial flora and nitrite formation in the healthy human stomach (Ger.). *Zbl. Bakt. Hyg. I. Abt. Orig. B.*, *178*, 297-315
- Mueller, R.L., Hagel, H.-J., Greim, G., Rupp, H. & Domschke, W. (1984) Dynamics of endogenous bacterial nitrite formation in the stomach. 1. Follow-up studies in humans under natural conditions (Ger.). *Zbl. Bakt. Hyg. I. Abt. Orig. B.*, *179*, 381-396
- Muir, C.S. & Shanmugaratnam, K. (1967) *The incidence of nasopharyngeal cancer in Singapore*. In: Muir, C.S. & Shanmugaratnam, K., eds, *Cancer of the Nasopharynx (UICC Monograph Series No. 1)*, Copenhagen, Munksgaard, pp. 47-53

- Mulder, G.J. & Scholtens, E. (1977) Phenolsulfotransferases and uridine diphosphate glucuronyltransferase from rat liver *in vivo* and *in vitro*. *Biochem. J.*, *165*, 553-559
- Murdia, U.S., Mehta, F.J. & Bhide, S.V. (1982) Nitrate reductase activity and nitrite levels in the saliva of habitual users of various tobacco products. *Food Chem. Toxicol.*, *20*, 269-271
- Muscroft, T.J., Deane, S.A., Young, D., Burdon, D.W. & Keighley, M. (1981) The microflora of the post operative stomach. *Br. J. Surg.*, *68*, 520-526
- Nagao, M., Wakabayashi, K., Fujita, Y., Tahira, T., Ochiai, M., Takayama, S. & Sugimura, T. (1986) *Nitrosatable precursors of mutagens in vegetables and soy sauce*. In: Hayashi, Y., Nagao, M., Sugimura, T., Takayama, S., Tomatis, L., Wattenberg, L.W. & Wogan, G.N., eds, *Diet, Nutrition and Cancer*, Tokyo/Utrecht, Japan Scientific Society Press/VNU Science Press, pp. 77-86
- Nair, J., Ohshima, H., Friesen, M., Croisy, A., Bhide, S.V. & Bartsch, H. (1985) Tobacco specific and betel nut specific N-nitroso compounds: occurrence in saliva and urine of betel quid chewers and formation *in vitro* by nitrosation of betel quid. *Carcinogenesis*, *6*, 295-303
- Nakamura, M., Osada, Y., Horiguchi, Y., Nakaoka, T., Ito, K. & Kawabata, T. (1985) Effect of megadoses of ascorbic acid on N-nitrosopiperidine metabolism by liver microsome (Jpn). *Bull. Kanagawa publ. Health Lab.*, *15*, 35-37
- Nakayama, T., Kimura, T., Kodama, M. & Nagata, C. (1983) Generation of hydrogen peroxide and superoxide anion from active metabolites of naphthylamines and aminoazo dyes: its possible role in carcinogenesis. *Carcinogenesis*, *4*, 765-769
- Napalkov, N. & Pozhariski, R.M. (1969) Morphogenesis of experimental tumors of the esophagus. *J. natl Cancer Inst.*, *42*, 927-931
- Narisawa, T., Hermanek, P., Habs, M. & Schmähl, D. (1984) Reduction of carcinogenicity of N-nitrosomethylurea by indomethacin and failure of resuming effect of prostaglandin E2 (PGE2) against indomethacin. *J. Cancer Res. clin. Oncol.*, *108*, 239-242
- National Research Council (1981) *The Health Effects of Nitrate, Nitrite and N-Nitroso Compounds, Part 1 of a 2-Part Study*, Washington DC, National Academy Press
- Nehls, P., Adamkiewicz, J. & Rajewsky, M.F. (1984) Immuno-slot-blot: a highly sensitive immunoassay for the quantitation of carcinogen-modified nucleosides in DNA. *J. Cancer Res. clin. Oncol.*, *108*, 23-29
- Neutel, C.I. & Buck, C. (1971) Effect of smoking during pregnancy on the risk of cancer in children. *J. natl Cancer Inst.*, *47*, 59-63
- Newberne, P.M. & Butler, W.H. (1969) Acute and chronic effects of aflatoxin B1 on the liver of domestic and laboratory animals: a review. *Cancer Res.*, *29*, 236-250
- Newell, G.R., Mansell, P.W., Spitz, M.R., Reuben, J.M. & Hersh, E.M. (1985) Volatile nitrites: use and adverse effects related to the current epidemic of the acquired immune deficiency syndrome. *Am. J. Med.*, *78*, 811-816
- Nordenstam, G.R., Ake Brandberg, C., Odén, A.S., Svanborg Edén, C.M. & Svanborg, A. (1986) Bacteriuria and mortality in an elderly population. *New Engl. J. Med.*, *314*, 1152-1156
- Norkus, E.P., Boyle, S., Kuenzig, W. & Mergens, W.J. (1984) Formation of N-nitrosomorpholine in mice treated with morpholine and exposed to nitrogen dioxide. *Carcinogenesis*, *5*, 549-554
- Northrop, D.B. (1982) Deuterium and tritium kinetic isotope effects on initial rates. *Meth. Enzymol.*, *87*, 607-625
- Nossal, N. & Hepple, H.A. (1966) Release of enzymes by osmotic shock from *Escherichia coli* in exponential phase. *J. biol. Chem.*, *241*, 3055
- Noyes, B.E. & Stark, G.R. (1975) Nucleic acid hybridization using DNA covalently coupled to cellulose. *Cell*, *5*, 301-310
- Ochiai, M., Wakabayashi, K., Nagao, M. & Sugimura, T. (1984) Tyramine is a major mutagen precursor in soy sauce, being convertible to a mutagen by nitrite. *Gann*, *75*, 1-3



- Ochhiai, M., Wakabayashi, K., Sugimura, T. & Nagao, M. (1986) Mutagenicities of indole and 30 derivatives after nitrite treatment. *Mutat. Res.* (in press)
- Office of Population Censuses and Surveys (1979) *Mortality Statistics, Area: England and Wales (DH5 No. 6)*, London
- Office of Prevention and Treatment of Cancer (1978) *Collection of Mortality Rates for Cancer in China*, Beijing, Ministry of Public Health
- Ohshima, H. (1983) GC-TEA determination of N-nitrosoproline in urine to provide an index for endogenous N-nitrosation. In: Preussmann, R., O'Neill, I.K., Eisenbrand, G., Spiegelhalter, B. & Bartsch, H., eds, *Environmental Carcinogens. Selected Methods of Analysis*, Vol. 6, N-Nitroso Compounds (IARC Scientific Publications No. 45), Lyon, International Agency for Research on Cancer, pp. 333-341
- Ohshima, H. & Bartsch, H. (1981) Quantitative estimation of endogenous nitrosation in humans by monitoring N-nitrosoproline excreted in the urine. *Cancer Res.*, *41*, 3658-3662
- Ohshima, H., Béréziate, J.-C. & Bartsch, H. (1982) Monitoring N-nitrosamino acids excreted in the urine and feces of rats as an index for endogenous nitrosation. *Carcinogenesis*, *3*, 115-120
- Ohshima, H., Friesen, M., O'Neill, I.K. & Bartsch, H. (1983) Presence in human urine of a new N-nitroso compound, N-nitrosothiazolidine 4-carboxylic acid. *Cancer Lett.*, *20*, 183-190
- Ohshima, H., O'Neill, I.K., Friesen, M., Béréziate, J.-C. & Bartsch, H. (1984a) Occurrence in human urine of new sulphur-containing N-nitrosamino acids N-nitrosothiazolidine 4-carboxylic acid and its 2-methyl derivative and their formation. *J. Cancer Res. clin. Oncol.*, *108*, 121-128
- Ohshima, H., O'Neill, I.K., Friesen, M., Pignatelli, B. & Bartsch, H. (1984b) Presence in human urine of new sulfur-containing N-nitrosamino acids: N-nitrosothiazolidine 4-carboxylic acid and N-nitroso 2-methylthiazolidine 4-carboxylic acid. In: O'Neill, I.K., von Borstel, R.C., Miller, C.T., Long, J. & Bartsch, H., eds, *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer (IARC Scientific Publications No. 57)*, Lyon, International Agency for Research on Cancer, pp. 77-85
- Ohshima, H., Muñoz, N., Nair, J., Calmels, S., Pignatelli, B., Crespi, M., Leclerc, H., Lu, S.H., Bhide, S.V., Vincent, P., Gounot, A.M. & Bartsch, H. (1985) Monitoring for endogenous formation of N-nitroso compounds by measurement of urinary N-nitrosamino acids. Its application to epidemiological and clinical studies. *Ann. Biol. Clin.*, *43*, 463-474
- Ohshima, H., Pignatelli, B., Nair, J., Muñoz, N., Calmels, S., Crespi, M., Lu, S.H., Bhide, S.V., Vincent, P., Leclerc, H., Kamiyama, S. & Bartsch, H. (1986) Urinary N-nitrosamino acids as indices of endogenous formation of N-nitroso compounds. In: Simic, N.G., Grossman, L. & Upton, A.C., eds, *Mechanisms of DNA Damage and Repair*, New York, Plenum Press, pp. 453-461
- Ohshima, H., Yang, H., Bourgade, M.C., Chen, J. & Bartsch, H. (1987) A correlation study on urinary excretion of N-nitroso compounds and oesophageal/gastric/liver cancer mortality in China. Methods of analysis of urine samples for nitrate, nitrite, N-nitroso compounds and thioethers. *Natl Cancer Inst. Monogr.* (in press)
- Ohta, T., Isa, M., Suzuki, Y., Yamahata, N., Suzuki, S. & Kurechi, T. (1981) Formation of mutagens from tryptophan by the reaction with nitrite. *Biochem. biophys. Res. Commun.*, *100*, 52-57
- Okada, M. (1984) Comparative metabolism of N-nitrosamines in relation to their organ and species specificity. In: O'Neill, I.K., von Borstel, R.C., Miller, C.T., Long, J. & Bartsch, H., eds, *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer (IARC Scientific Publications No. 57)*, Lyon, International Agency for Research on Cancer, pp. 401-409
- Okada, M. & Hashimoto, Y. (1974) Carcinogenic effect of N-nitrosamines related to butyl(4-hydroxybutyl)nitrosamine in ACI/N rats, with special reference to induction of urinary bladder tumors. *Gann*, *65*, 13-19
- Okada, M. & Ishidate, M. (1977) Metabolic fate of N-n-butyl-N-(4-hydroxybutyl)nitrosamine and its analogues. Selective induction of urinary bladder tumours in the rat. *Xenobiotica*, *7*, 11-24

- Okada, M., Suzuki, E. & Hashimoto, Y. (1976) Carcinogenicity of *N*-nitrosamines related to *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine and *N,N*-dibutylnitrosamine in ACl/N rats. *Gann*, *67*, 825-834
- Okada, M., Mochizuki, M., Anjo, T., Sone, T., Wakabayashi, Y. & Suzuki, E. (1980) Formation, deoxygenation and mutagenicity of  $\alpha$ -hydroperoxydialkylnitrosamines. In: Walker, E.A., Castegnaro, M., Gričič, L. & Börzsönyi, M., eds, *N-Nitroso Compounds: Analysis, Formation and Occurrence (IARC Scientific Publications No. 31)*, Lyon, International Agency for Research on Cancer, pp. 71-82
- Okamoto, T., Isogai, Y., Koizumi, T., Fujishiro, H. & Sato, Y. (1967) Studies on plant growth regulators. III. Isolation of indole-3-acetonitrile and methyl indole-3-acetate from the neutral fraction of the Moyashi extract. *Chem. Pharm. Bull.*, *15*, 163-168
- Okamura, M. (1981) A specific method for determination of total ascorbic acids in urine by the  $\alpha,\alpha'$ -dipyridyl method. *Clin. chim. Acta*, *115*, 393-403
- Olsson, U., Onfelt, A. & Beije, B. (1984) Dietary selenium deficiency causes decreased *N*-oxygenation of *N,N*-dimethylaniline and increased mutagenicity of dimethylnitrosamine in the isolated rat liver/cell culture system. *Mutat. Res.*, *126*, 73-80
- Omaye, S.T. & Turnbull, J.D. (1979) Degradation of cytochrome P-450 heme in ascorbic acid-deficient guinea pigs. *Biochem. Pharmacol.*, *28*, 3651-3657
- Omura, T. & Sato, R. (1964) The carbon monoxide binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J. biol. Chem.*, *193*, 2370-2378
- Otani, S., Matsui, I., Kuramoto, A. & Marisawa, S. (1984) Induction of ornithine decarboxylase in guinea-pig lymphocytes and its relation to phospholipid metabolism. *Biochim. biophys. Acta*, *800*, 96-101
- Pacifici, G.M., Richter, E., Lehmann, G., Wiessler, M., Zwicklenpflug, W. & Giuliani, L. (1986) Hydroxylation of dibutylnitrosamine in the human liver and intestinal microsomal fractions. *Arch. Toxicol.*, *58*, 196-198
- Palladino, G., Adams, J.D., Brunneemann, K.D., Haley, N.J. & Hoffmann, D. (1986) Snuff-dipping in college students: a clinical profile. *Military Med.*, *151*, 342-346
- Panalaks, T., Iyengar, J.R. & Sen, N.P. (1973) Nitrate, nitrite, and dimethylnitrosamine in cured meat products. *J. Assoc. off. anal. Chem.*, *56*, 621-625
- Panalaks, T., Iyengar, J.R., Donaldson, B.A., Miles, W.F. & Sen, N.P. (1974) Further survey of cured meat products for volatile *N*-nitrosamines. *J. Assoc. off. anal. Chem.*, *57*, 806-812
- Pani, B., Badurdi, N., Bartoli-Klugmann, F., Venturini, S. & di Fant, I. (1980) Mutagenic activity of 3-diazoacetyl-glycine derivatives on V79 Chinese hamster cells. *Mutat. Res.*, *78*, 375-379
- Parodi, S., Taningher, M. & Santi, L. (1982) Alkaline elution in vivo: fluorometric analysis in rats. Quantitative predictivity of carcinogenicity, as compared with other short-term tests. In: Nicolini, C., ed., *Chemical Carcinogenesis*, New York, Plenum, pp. 137-155
- Parsa, I., Marsh, W.H., Sutton, A.L. & Butt, K.M.H. (1981) Effects of dimethylnitrosamine on organ-cultured adult human pancreas. *Am. J. Pathol.*, *72*, 403-411
- Patel, D.J., Shapiro, L., Kozlowski, S.A., Gaffney, B.L. & Jones, R.A. (1986a) Structural studies of the O<sup>6</sup>-meG<sup>+</sup>C interaction in the d(C-G-C-G-A-A-T-T-C-O<sup>6</sup>meG-C-G) duplex. *Biochemistry*, *25*, 1027-1036
- Patel, D.J., Shapiro, L., Kozlowski, S.A., Gaffney, B.L. & Jones, R.A. (1986b) Structural studies of the O<sup>6</sup>meG<sup>+</sup>T interaction in the d(C-G-C-G-A-A-T-T-C-O<sup>6</sup>meG-C-G) duplex. *Biochemistry*, *25*, 1036-1042
- Patten, C., Ning, S.M., Lu, A.Y.H. & Yang, C.S. (1986a) Acetone induced P450: purification, catalytic activity, and interactions with cytochrome b<sub>5</sub>. *Fed. Proc.*, *45*, 1078
- Patten, C., Ning, S.M., Lu, A.Y.H. & Yang, C.S. (1986b) Acetone-inducible cytochrome P-450: purification, catalytic activity, and interaction with cytochrome b<sub>5</sub>. *Arch. Biochem. Biophys.* (in press)
- Paul, A.A. & Southgate, D.A.T. (1978) *McCance and Widdowson's The Composition of Foods*, London, Her Majesty's Stationary Office

- Pegg, A.E. (1980) *Metabolism of N-nitrosodimethylamine*. In: Montesano, R., Bartsch, H. & Tomatis, L., eds, *Molecular and Cellular Aspects of Carcinogen Screening Tests (IARC Scientific Publications No. 27)*, Lyon, International Agency for Research on Cancer, pp. 3-22
- Pegg, A.E. (1983) Alkylation and subsequent repair of DNA after exposure to dimethylnitrosamine and related carcinogens. *Rev. Biochem. Toxicol.*, 5, 83-133
- Pegg, A.E. (1984) Methylation of the O<sup>6</sup>-position of guanine in DNA is the most likely initiating event in carcinogenesis by methylating agents. *Cancer Invest.*, 2, 223-231
- Pegg, A.E. (1986) *Factors affecting O<sup>6</sup>-alkylguanine-DNA-alkyltransferase activity*. In: Hoffmann, D. & Harris, C.C., eds, *Mechanisms of Tobacco Carcinogenesis (Banbury Report 23)*, Cold Spring Harbor, NY, CSH Press, pp. 287-298
- Pegg, A.E., Wiest, L., Foote, R.S., Mitra, S. & Perry, W. (1983) Purification and properties of O<sup>6</sup>-methylguanine-DNA transmethylase from rat liver. *J. Biol. Chem.*, 258, 2327-2334
- Pegg, A.E., Schicchitano, D. & Dolan, M.E. (1984) Comparison of rates of repair of O<sup>6</sup>-alkylguanines in DNA by rat liver and bacterial O<sup>6</sup>-alkylguanine-DNA alkyltransferase. *Cancer Res.*, 44, 3806-3811
- Peng, R., Tu, Y.Y. & Yang, C.S. (1982) The induction and competitive inhibition of a high affinity microsomal nitrosodimethylamine demethylase by ethanol. *Carcinogenesis*, 3, 1457-1461
- Pensabene, J.W., Gates, R.A., Jenkins, R.K. & Fiddler, W. (1986) Effect of <sup>137</sup>-cesium radiation on the formation of N-nitrosopyrrolidine in bacon. *J. Agric. Food Chem.* (in press)
- Peraino, C., Richards, W.L. & Stevens, F.J. (1983) *Multistage hepatocarcinogenesis*. In: Slaga, T., ed., *Mechanisms of Tumor Promotion*, Vol. 1, Boca Raton, FL, CRC Press, pp. 1-53
- Perera, F.P., Poirier, M.C., Yuspa, S.H., Nakayama, J., Jeretzki, A., Curnen, M.M., Knowles, D.M. & Weinstein, I.B. (1982) A pilot project in molecular cancer epidemiology; determination of benzo[a]pyrene-DNA adducts in animal and human tissues by immunoassays. *Carcinogenesis*, 3, 1405-1410
- Pesek, J.J. (1978) Quantitative determination of cis:trans isomeric ratios of substituted thiazolidines by carbon-13 magnetic resonance spectrometry. *Anal. Chem.*, 50, 787-789
- Peters, J.M., Preston-Martin, S. & Yu, M.C. (1981) Brain tumors in children and occupational exposure of parents. *Science*, 213, 235-237
- Peto, R., Gray, R., Brantom, P. & Grasso, P. (1984) *Nitrosamine carcinogenesis in 5120 rodents: chronic administration of sixteen different concentrations of NDEA, NDMA, NPYR and NPIP in the water of 4440 inbred rats with parallel studies on NDEA alone on the effect of age starting (3, 6 or 20 weeks) and of species (rats, mice or hamsters)*. In: O'Neill, I.K., von Borstel, R.C., Miller, C.T., Long, J. & Bartsch, H., eds, *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer (IARC Scientific Publications No. 57)*, Lyon, International Agency for Research on Cancer, pp. 627-665
- Pettigrew, A.R. & Fell, G. (1972) Simplified colorimetric determination of thiocyanate in biological fluids and its application to investigation of the toxic amblyopias. *Clin. Chem.*, 18, 966-1000
- Pfab, J., Häger, J. & Krieger, W. (1983) Energy partitioning in the 532 nm photofragmentation of nitrosyl cyanide (NCNO). *J. Chem. Phys.*, 78, 266-274
- Phillips, J.C., Heading, C.E., Lake, B.G., Gangoli, S.D. & Lloyd, A.G. (1974) Studies *in vivo* on the inhibition of dimethylnitrosamine metabolism in the rat. *Biochem. Soc. Trans.*, 2, 885-889
- Phillips, J.C., Bex, C., Lake, B.G., Cettrell, R.C. & Gangoli, S.D. (1982) Inhibition of dimethylnitrosamine metabolism by some heterocyclic compounds and by substrates and inhibitors of monoamine oxidase in the rat. *Cancer Res.*, 42, 3761-3765
- Phizackerley, P.J.R. & Al-Dabbagh, S.A. (1983) The estimation of nitrate and nitrite in saliva and urine. *Anal. Biochem.*, 130, 242-245
- Pignatelli, B., Béréziat, J.-C., Descote, G. & Bartsch, H. (1982) Catalysis of nitrosation *in vitro* and *in vivo* in rats by catechin and resorcinol and inhibition by chlorogenic acid. *Carcinogenesis*, 3, 1045-1049

- Pignatelli, B., Scriban, R., Descotes, G. & Bartsch, H. (1984) Modifying effects of polyphenols and other constituents of beer on the formation of N-nitroso compounds. *Am. Soc. Brewing Chem. J.*, *42*, 18-23
- Pignatelli, B., Malaville, C., Friesen, M., Hautefeuille, A., Bartsch, H. & Piskorska, D. (1987) Synthesis, structure-activity relationships and a reaction mechanism for mutagenic N-nitroso derivatives of glycosylamines and Amadori compounds: model substances for N-nitrosated early Maillard reaction products. (submitted for publication)
- Pipkin, G.E., Schlegel, J.U., Nishimura, R. & Shultz, G.N. (1969) Inhibitory effect of L-ascorbate on tumor formation in urinary bladder implanted with 3-hydroxyxanthranilic acid. *Proc. Soc. exp. Biol. Med.*, *131*, 522-524
- Piszkiwicz, D. (1977) *Kinetics of Chemical and Enzyme-catalyzed Reactions*, New York, Oxford University Press, pp. 83-89
- Pitot, H.C. & Sirica, A.E. (1980) The stages of initiation and promotion in hepatocarcinogenesis. *Biochim. biophys. Acta*, *605*, 191-215
- Pliss, G.B., Zabezhinski, M.A., Petrov, A.S. & Khudoley, V.V. (1982) Peculiarities of N-nitramines carcinogenic action. *Arch. Geschwulstforsch.*, *52*, 629-634
- Pollock, J.R.A. (1985) Formation of nitrosoiminodialkanoic acids during the nitrosation of peptides. *Food Chem. Toxicol.*, *23*, 701-704
- Pool, B.L. & Wiessler, M. (1981) Investigations on the mutagenicity of primary and secondary  $\alpha$ -acetoxynitrosamines with *Salmonella typhimurium*: activation and deactivation of structurally related compounds by S9. *Carcinogenesis*, *2*, 991-997
- Pool, B.L., Frei, E., Plesch, W.J., Romruen, K. & Wiessler, M. (1984a) Formaldehyde as a possible mutagenic metabolite of N-nitrodimethylamine and other agents which are suggested to yield non-alkylating species *in vitro*. *Carcinogenesis*, *5*, 809-814
- Pool, B.L., Röper, H., Röper, S. & Romruen, K. (1984b) Mutagenic studies on N-nitrosated products of the Maillard browning reaction: N-nitrosofructose-aminoacids. *Food Chem. Toxicol.*, *22*, 797-801
- Port, C.J. & Thurnam, D.I. (1983) Erythropoiesis and red cell life span in riboflavin-deficient rats. *Proc. Nutr. Soc.*, *42*, 116A
- Pour, P., Krüger, F.W., Cardesa, A., Althoff, J. & Mohr, U. (1973) Carcinogenic effect of d-n-propylnitrosamine in Syrian golden hamsters. *J. natl Cancer Inst.*, *51*, 1019-1027
- Pour, P., Krüger, F.W., Althoff, J., Cardesa, A. & Mohr, U. (1974) The effect of beta-oxidized nitrosamines on Syrian golden hamsters. 3. 2,2'-Dihydroxy-di-n-propylnitrosamine. *J. natl Cancer Inst.*, *54*, 141-146
- Pour, P., Salmasi, S. & Runge, R. (1978) Selective induction of pancreatic adenocarcinomas in the Syrian hamster by a single dose of N-nitrosobis(2-oxopropyl)amine. *Cancer Lett.*, *4*, 317-321
- Pour, P., Runge, R., Birt, D., Gingell, R., Lawson, T., Nagel, D. & Wallcave, R. (1981) Current knowledge of pancreatic carcinogenesis and its relevance to the human disease. *Cancer*, *47*, 1573-1584
- Povey, A.C. (1985) *The Preparation and Use of Semi-permeable Microcapsules Containing Macromolecular Nucleophiles in the In-vitro and In-vivo Entrapment of Carcinogens*, PhD Thesis, University of London
- Povey, A.C., Nixon, J.R., Bartsch, H. & O'Neill, I.K. (1986) *Preparation and use of semi-permeable magnetic microcapsules for the in-vitro and in-vivo trapping of carcinogens*. In: *Proceedings of the 4th International Conference on Pharmaceutical Technology, Paris, 1986*, Vol. IV, Chateau Malabry, Association de Pharmacie Galénique Industrielle, pp. 140-148
- Prasanna, H.R., Lotlikar, P.D., Brandt, C. & Magee, P.N. (1985) Factors influencing the activity of dimethylnitrosamine demethylase in hamster, rat and chicken liver microsomes. *Cancer Lett.*, *26*, 25-32

- Préat, V., de Gerlache, J., Lans, M., Taper, H. & Roberfroid, M. (1986) Influence of the nature and the dose of the initiator on the development of premalignant and malignant lesions in rat hepatocarcinogenesis. *Teratog. Carcinog. Mutagen.*, **6**, 165-172
- Preston, B.D., Singer, B. & Loeb, L.A. (1986a) Site-specific, polymerase-catalyzed insertion of alkyl-deoxyribonucleoside triphosphate adducts in DNA. In: Ramel, C., Lambert, B. & Magnusson, J., eds, *Genetic Toxicology of Environmental Chemicals*, New York, Alan R. Liss
- Preston, B.D., Singer, B. & Loeb, L.A. (1986b) Mutagenic potential of O<sup>6</sup>-methylthymine *in vivo* determined by an enzymatic approach to site-specific mutagenesis. *Proc. natl Acad. Sci. USA* (in press)
- Preston-Martin, S. & Henderson, B.E. (1984) N-Nitroso compounds and human intracranial tumors. In: O'Neill, I.K., von Borstel, R.C., Miller, C.T., Long, J. & Bartsch, H., eds, *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer (IARC Scientific Publications No. 57)*, Lyon, International Agency for Research on Cancer, pp. 887-894
- Preston-Martin, S., Yu, M.C., Benton, B. & Henderson, B.E. (1982) N-Nitroso compounds and childhood brain tumors: a case-control study. *Cancer Res.*, **42**, 5240-5245
- Preussmann, R. (1980) Dose-response studies and 'no-effect-levels' of N-nitroso compounds. *Oncology*, **37**, 243-250
- Preussmann, R., ed. (1983) *Das Nitrosamin-Problem*, Weinheim, Verlag Chemie
- Preussmann, R. (1984) Carcinogenic N-nitroso compounds and their environmental significance. *Naturwissenschaften*, **71**, 25-30
- Preussmann, R. & Eisenbrand, G. (1984) N-Nitroso carcinogens in the environment. In: Searle, C.E., ed., *Chemical Carcinogens (ACS Symposium Series 182)*, Washington DC, American Chemical Society, pp. 829-868
- Preussmann, R. & Stewart, B.W. (1984) N-Nitroso carcinogens. In: Searle, C.E., ed., *Chemical Carcinogens (ACS Symposium Series 182)*, Washington DC, American Chemical Society, pp. 643-828
- Preussmann, R., Schmähl, D. & Eisenbrand, G. (1977) Carcinogenicity of N-nitrosopyrrolidine: dose response study in rats. *Z. Krebsforsch.*, **90**, 161-166
- Preussmann, R., Habs, M., Pool, B., Stummeyer, D., Lijinsky, W. & Reuber, M. (1981) Fluoro-substituted N-nitrosamines. I. Inactivity of N-nitroso-bis(2,2,2-trifluoroethyl)amine in carcinogenicity and mutagenicity tests. *Carcinogenesis*, **2**, 753-756
- Preussmann, R., Habs, M., Habs, H. & Schmähl, D. (1982) Carcinogenicity of N-nitrosodiethanolamine in rats at five different dose levels. *Cancer Res.*, **42**, 5167-5171
- Prival, M.J. & Mitchell, V.D. (1981) Influence of microsomal and cytosolic fractions from rat, mouse, and hamster liver on the mutagenicity of dimethylnitrosamine in the *Salmonella* plate incorporation assay. *Cancer Res.*, **41**, 4361-4367
- Prokopczyk, B., Rivenson, A., Bertinato, P., Brunnemann, K.D. & Hoffmann, D. (1987) A study of betel quid carcinogenesis. V. 3-(Methylnitrosamino)propionitrile: occurrence in saliva, carcinogenicity and DNA methylation in F344 rats. *Cancer Res.*, **47** (in press)
- Pryor, W.A., Castle, L. & Church, D.F. (1985) Nitrosation of organic hydroperoxides by nitrogen dioxide/dinitrogen tetroxide. *J. Am. chem. Soc.*, **107**, 211-217
- Pulciani, S., Santos, E., Long, L.K., Sorrentino, V. & Barbacid, M. (1985) *ras* Gene amplification and malignant transformation. *Mol. cell. Biol.*, **5**, 2836-2841
- Pylypiw, H.M., Zubroff, J.R., Magee, P.N. & Harrington, G.W. (1984) The metabolism of N-nitrosomethylaniline. *J. Cancer Res. clin. Oncol.*, **108**, 66-70
- Pylypiw, H.M., Jr, Zimmerman, F., Harrington, G.W. & Anderson, L.M. (1985) Apparatus for trace determination of volatile N-nitrosamines in small samples. *Anal. Chem.*, **57**, 2996-2997
- Quan, P.M., Karns, T.K.B. & Quin, L.D. (1965) The synthesis of anatabine and related compounds. *J. org. Chem.*, **30**, 2769-2772

- Rabes, H.M., Bücher, T., Hartmann, A., Linke, I. & Dünwald, M. (1982) Clonal growth of carcinogen-induced enzyme-deficient preneoplastic cell populations in mouse liver. *Cancer Res.*, **42**, 3220-3227
- Radomski, J.L., Greenwald, D., Hearn, W.L., Block, N.L. & Woods, F.M. (1978) Nitrosamine formation in bladder infections and its role in the etiology of bladder cancer. *J. Urol.*, **120**, 48-50
- Ralt, D. & Tannenbaum, S.R. (1981) *The role of bacteria in nitrosamine formation*. In: Scanlan, R.A. & Tannenbaum, S.R., eds, *N-Nitroso Compounds (ACS Symposium Series 174)*, Washington DC, American Chemical Society, pp. 159-164
- Rao, T.K., Hardigree, A.A., Young, J.A., Lijinsky, W. & Epler, J.L. (1977) Mutagenicity of N-nitrosopiperidines with *Salmonella typhimurium*/microsomal activation system. *Mutat. Res.*, **56**, 131-145
- Reddy, B.S., Hirota, N. & Katayama, S. (1982) Effect of dietary sodium ascorbate on 1,2-dimethylhydrazine or methylnitrosourea-induced colon carcinogenesis in rats. *Carcinogenesis*, **3**, 1097-1099
- Reed, P.I. (1986) The role of nitrosamines in cancer formation. *Bibliotheca Nutr. Dieta*, **37**, 130-138
- Reed, P.I., Smith, P.L., Haines, K., House, F.R. & Walters, C.L. (1981a) Gastric juice N-nitrosamines in health and gastroduodenal disease. *Lancet*, *ii*, 550-552
- Reed, P.I., Smith, P.L.R., Haines, K., House, F.R. & Walters, C.L. (1981b) The effect of cimetidine on gastric N-nitrosamine concentration. *Lancet*, *ii*, 553-556
- Reese, C.B. & Skone, P.A. (1984) The protection of thymine and guanine residues in oligodeoxyribonucleotide synthesis. *J. chem. Soc. Perkin Trans.*, **1**, 1263-1271
- Reeve, A.E. & Hopkins, T.R. (1980) Photochemical reaction between protein and nucleic acids: photoaddition of tryptophan to pyrimidine bases. *Photochem. Photobiol.*, **31**, 297
- Reichart, P., Böning, W., Srisuwan, S., Theetramont, C. & Mohr, U. (1984) Ultrastructural findings in the oral mucosa of betel chewers. *J. oral Pathol.*, **13**, 166-177
- Reinacher, M., Eigenbrodt, E., Gerbracht, U., Zenk, G., Timmermann-Trosiener, I., Bentley, P., Waechter, F., Schoner, W. & Schulte-Hermann, R. (1986) Pyruvate kinase isoenzymes in altered foci and carcinoma of rat liver. *Carcinogenesis*, **7**, 1351-1357
- Reinke, L.A., Kauffman, F.C., Belinsky, S.A. & Thurman, R.G. (1983) Effects of ethanol and sorbitol on mixed-function oxidation in perfused rat livers. *Pharmacol. Biochem. Behav.*, **18**, 289-294
- Reynolds, J.E.F., ed. (1982) *Martindale — The Extra Pharmacopoea*, 28th ed., London, The Pharmaceutical Press
- Reznik-Schüller, H. & Reznik, G. (1979) Experimental pulmonary carcinogenesis. *Int. Rev. exp. pathol.*, **20**, 211-281
- Reznik-Schüller, H.M. & Hague, B.F., Jr (1981) Autoradiographic study of the distribution of bound radioactivity in the respiratory tract of Syrian hamsters given N-[<sup>3</sup>H]nitrosodiethylamine. *Cancer Res.*, **41**, 2147-2150
- Rhim, J.S., Fujita, J., Arnstein, P. & Aaronson, A.A. (1986) Neoplastic conversion of human epidermal keratinocytes by adenovirus 12-SV-20 virus and chemical carcinogens. *Science*, **232**, 385-387
- Rice, J.M. & Ward, J.M. (1982) Age dependence of susceptibility to carcinogenesis in the nervous system. *Ann. N.Y. Acad. Sci.*, **381**, 274-289
- Richardson, F.C., Dyroff, M.C., Boucheron, J.A. & Swenberg, J.A. (1985) Differential repair of O<sup>6</sup>-alkylthymidine following exposure to methylating and ethylating hepatocarcinogens. *Carcinogenesis*, **6**, 625-629
- Richardson, F.C., Boucheron, J.A., Dyroff, M.C., Popp, J.A. & Swenberg, J.A. (1986) Biochemical and morphologic studies of heterogeneous lobe responses in hepatocarcinogenesis. *Carcinogenesis*, **7**, 247-251
- Richter, E., Richter-Cooberg, U., Feng, X., Schulze, J. & Wiessler, M. (1986) Intestinal metabolism of nitrosamines. 1. Transport and metabolism of six nitrosamines in isolated perfused rat small intestinal segments. *Carcinogenesis*, **7**, 1207-1213

- Rickard, R.W. & Dorough, H.W. (1984) In vivo formation of nitrosocarbamates in the stomach of rats and guinea-pigs. *J. Toxicol. environ. Health*, *14*, 279-290
- Ridd, J.H. (1961) Nitrosation, diazotisation and deamination. *Q. Rev.*, *15*, 418-441
- Ringler, D.H. & Dabich, L. (1979) *Hematology and clinical biochemistry*. In: Baker, H.J., Lindsey, J.R. & Weisbroth, S.H., eds, *The Laboratory Rat*, Vol. 1, New York, Academic Press, pp. 105-121
- Roellig, M.P., Houston, P.L., Asscher, M. & Haas, Y. (1980) CF<sub>3</sub>NO photodissociation dynamics. *J. Chem. Phys.*, *73*, 5081-5091
- Roos, D.S. & Choppin, P.W. (1984) Tumorigenicity of cell lines with altered lipid composition. *Proc. natl Acad. Sci. USA*, *81*, 7622-7626
- Röper, H., Röper, S. & Heyns, K. (1982) N-Nitroso sugar amino acids (N-NO-D-fructose-L-amino acids). In: Bartsch, H., O'Neill, I.K., Castegnaro, M. & Okada, M., eds, *N-Nitroso Compounds: Occurrence and Biological Effects (IARC Scientific Publications No. 41)*, Lyon, International Agency for Research on Cancer, pp. 87-98
- Röper, H., Röper, S. & Meyer, B. (1984) Amadori- and N-nitroso-Amadori compounds and their pyrolysis products. *Chemical, analytical and biological aspects*. In: O'Neill, I.K., von Borstel, R.C., Miller, C.T., Long, J. & Bartsch, H., eds, *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer (IARC Scientific Publications No. 57)*, Lyon, International Agency for Research on Cancer, pp. 101-111
- Ross, H.D. & Hotchkiss, J.H. (1985) Determination of nitrate in dried foods by gas chromatography-thermal energy analyzer. *J. Assoc. off. anal. Chem.*, *68*, 41-43
- Rowland, I.R. & Grasso, P. (1975) Degradation of N-nitrosamines by intestinal bacteria. *Appl. Microbiol.*, *29*, 7-12
- Ruddell, W.S.J., Bone, E.S., Hill, M.J. & Walters, C.L. (1978) Pathogenesis of gastric cancer in pernicious anaemia. *Lancet*, *i*, 521-523
- Russell, G.F. (1983) Nitrite interactions in model Maillard browning systems. In: Waller, G.R. & Feather, M.S., eds, *The Maillard Reaction in Foods and Nutrition (ACS Symposium Series 215)*, Washington DC, American Chemical Society, pp. 83-90
- Ryan, D.E., Ramanathan, L., Iida, S., Thomas, P.E., Haniu, M., Shively, J.E., Lieber, C.S. & Levin, W. (1985) Characterization of a major form of rat hepatic microsomal cytochrome P-450 induced by isoniazid. *J. biol. Chem.*, *260*, 6385-6393
- Ryan, D.E., Koop, D.R., Thomas, P.E., Coon, M.J. & Levin, W. (1986) Evidence that isoniazid and ethanol induce the same microsomal cytochrome P-450 in rat liver, an isozyme homologous to rabbit liver cytochrome P-450 isozyme 3a. *Arch. Biochem. Biophys.*, *246*, 633-644
- Saffhill, R. (1985) In vitro miscoding of alkylthymines with DNA and RNA polymerases. *Chem.-biol. Interactions*, *53*, 121-130
- Saffhill, R., Margison, G.P. & O'Connor, P.J. (1985) Mechanism of carcinogenesis induced by alkylating agents. *Biochim. biophys. Acta*, *823*, 111-145
- Saffiotti, V., Cefis, F. & Kobb, L.H. (1968) A method for the experimental induction of bronchogenic carcinoma. *Cancer Res.*, *28*, 104-124
- Sakshaug, J., Sjøgnen, E., Aas Hansen, M. & Koppang, N. (1965) Dimethylnitrosamine, its hepatotoxic effect in sheep and its occurrence in toxic batches of herring meal. *Nature*, *206*, 1261-1262
- Sander, J. & Bürkle, G. (1969) Induction of malignant tumors in rats by simultaneous feeding of nitrite and secondary amines (Ger.). *Z. Krebsforsch.*, *76*, 93-96
- Sander, J., Schweinsberg, F., LaBar, J., Bürkle, G. & Schweinsberg, E. (1975) Nitrite and nitrosable amino compounds in carcinogenesis. *Gann. Monogr.*, *17*, 145-160
- Sandler, D.P., Everson, R.B., Wilcox, A.J. & Browder, J.P. (1985) Cancer risk in adulthood from early life exposure to parents' smoking. *Am. J. publ. Health*, *75*, 487-492

- Santamaria, L., Bianchi, A., Arnaboldi, A., Andreoni, L., Ravetto, G., Santagati, G. & Bermond, P. (1986) *Chemoprevention by supplemental carotenoids of skin, mammary and gastric experimental carcinogenesis. Perspectives in human clinical trials*. In: *Proceedings of the 14th International Cancer Congress*, Basel, Karger, Abstract No. 3258
- Sasaki, K., Imamura, K. & Tanaka, T. (1983) Mechanism of hepatic ornithine decarboxylase induction by the ornithine decarboxylase-inducing factor isolated from tumor ascites fluid: determination of target cells for the factor in the liver. *J. Biochem.*, **94**, 949-959
- Sato, S., Tadenuma, M., Takahashi, K. & Nakamura, N. (1975) Configuration of sake taste. VI. Rough taste components. 3. Tetrahydroharman-3-carboxylic acid. *Nippon Jozo Kyokai Zasshi*, **70**, 821-824
- Saul, R.L. & Archer, M.C. (1983) Nitrate formation in rats exposed to nitrogen dioxide. *Toxicol. appl. Pharmacol.*, **67**, 284-291
- Saul, R.L. & Archer, M.C. (1984a) Oxidation of ammonia and hydroxylamine to nitrate in the rat and *in vitro*. *Carcinogenesis*, **5**, 77-81
- Saul, R.L. & Archer, M.C. (1984b) *Oxidation of ammonia and hydroxylamine to nitrate in the rat*. In: O'Neill, I.K., von Borstel, R.C., Miller, C.T., Long, J. & Bartsch, H., eds, *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer (IARC Scientific Publications No. 57)*, Lyon, International Agency for Research on Cancer, pp. 241-246
- Savel, J. & Prokopova, M. (1982) Formation of nitrites in contaminated brewer's yeast. *Kvasny Prumysl.*, **28**, 128-131
- Sayer, J.M., Yagi, H., Wood, A.W., Conney, A.H. & Jerina, D.M. (1982) Extremely facile reaction between the ultimate carcinogen benzo[a]pyrene-7,8-diol 9,10-epoxide and ellagic acid. *J. Am. chem. Soc.*, **104**, 5562-5564
- Scanlan, R. (1983) Formation and occurrence of nitrosamines in foods. *Cancer Res.*, **43**, 2435-2440
- Scherer, E. (1984) Neoplastic progression in experimental hepatocarcinogenesis. *Biochim. biophys. Acta*, **738**, 219-236
- Scheunig, G. & Ziebarth, D. (1978) Formation of N-nitroso compounds from drugs and nitrite in human gastric juice *in vitro* (Ger.). *Pharmazie*, **33**, 722-726
- Schirmer, R.H., Lederbogen, F., Krauth-Siegel, R.L., Eisenbrand, G., Schulz, G. & Jung, A. (1984) *Flavoenzymes as drug targets*. In: Bray, R.C., Engel, P.C. & Mayhew, S.G., eds, *Flavins and Flavoproteins*, Berlin (West), W. de Gruyter, pp. 847-859
- Schlag, P., Ulrich, H., Merkle, P., Böckler, R., Peter, M. & Herfarth, C. (1980) Are nitrite and N-nitroso compounds in gastric juice risk factors for carcinoma in the operated stomach? *Lancet*, **i**, 727-729
- Schmähl, D. (1970) Experimental investigations on syncarcinogenesis. VI. Addition of minimum doses of four different hepatotropic carcinogens in hepatocarcinogenesis in rats (Ger.). *Z. Krebsforsch.*, **74**, 457-466
- Schmähl, D. (1976) Investigations on oesophageal carcinogenicity by methyl-phenyl-nitrosamine and ethyl alcohol in rats. *Cancer Lett.*, **1**, 215-218
- Schmähl, D. (1979) *N-Nitroso compounds as potential carcinogens in our environment*. (Ger.) In: *19th Annual Meeting of the German Society for Social Medicine*, Stuttgart, A.W. Gentner, pp. 233-237
- Schmähl, D. (1981) *The role of nitrosamines in carcinogenesis*. In: Gibson, C.G., Joannides, C. & Taylor, A.B., eds, *Safety Evaluations of Nitrosatable Drugs and Chemicals*, London, Pergamon Press, pp. 1-11
- Schmähl, D. & Habs, M. (1978) Experiments on the influence of an aromatic retinoid on the chemical carcinogenesis in rats by butyl-butanol-nitrosamine and 1,2-dimethylhydrazine. *Arzneimittel.-Forsch.*, **28**, 49-51
- Schmähl, D. & Habs, M. (1981) Experiments on the influence of an aromatic retinoid on the chemical carcinogenesis induced by N-nitroso-methyl benzylamine in rats. *Arzneimittel.-Forsch.*, **31**, 677-679



- Schmähl, D. & Krüger, F.W. (1972) Lack of syncarcinogenic effect of cyclamate in bladder carcinogenesis induced by butyl-butanol-nitrosamine in rats (Ger.). *Arzneimittel.-Forsch.*, **22**, 999-1000
- Schmähl, D. & Thomas, C. (1962) Experiments on carcinogenesis induced by concomitant administration of 4-dimethylamino-azobenzene and diethylnitrosamine in rats (Ger.). *Naturwissenschaften*, **49**, 521-522
- Schmähl, D. & Thomas, C. (1963) Carcinogenesis induced by administration of diethylnitrosamine and X-rays in rats (Ger.) *Naturwissenschaften*, **50**, 308-309
- Schmähl, D. & Thomas, C. (1965) Experimental investigations on syncarcinogenesis. IV. Experiments on carcinogenesis induced by concomitant oral administration of diethylnitrosamine and 4-dimethylaminostilbene in rats (Ger.). *Z. Krebsforsch.*, **67**, 135-140
- Schmähl, D., Thomas, C. & Brune, H. (1964) Experimental investigations on syncarcinogenesis. II. Experiments on carcinogenesis induced by concomitant administration of urethan and 9,10-dimethyl-1,2-benzanthracene in mice (Ger.). *Z. Krebsforsch.*, **66**, 297-302
- Schmähl, D., Thomas, C., Sattler, W. & Scheld, G.F. (1965) Experimental investigations on syncarcinogenesis. III. Experiments on carcinogenesis induced by concomitant administration of diethylnitrosamine and carbon tetrachloride or ethanol in rats; also an experimental contribution to 'alcohol cirrhosis' (Ger.). *Z. Krebsforsch.*, **66**, 526-532
- Schmähl, D., Wagner, R. & Scherf, H.R. (1971) Influence of immunosuppressive agents on carcinogenesis by diethylnitrosamine in rat liver and on sarcoma formation by benzo(a)pyrene (Ger.). *Arzneimittel. Forsch.*, **21**, 403-404
- Schmähl, D., Mundt, D. & Schmidt, F.G. (1974) Experimental investigations on the influence upon the chemical carcinogenesis. I. Studies with ethylnitrosourea. *Z. Krebsforsch.*, **82**, 91-100
- Schmähl, D., Danisman, A., Habs, M. & Diehl, B. (1976a) Experimental investigations on the influence upon the chemical carcinogenesis. Studies with 1,2-dimethylhydrazine. *Z. Krebsforsch.*, **86**, 89-94
- Schmähl, D., Krüger, F.W., Habs, M. & Diehl, B. (1976b) Influence of disulfiram on the organotropy of the carcinogenic effect of dimethylnitrosamine and diethylnitrosamine in rats. *Z. Krebsforsch.*, **85**, 271-276
- Schmähl, D., Habs, M. & Diehl, B. (1976c) Experimental investigations on the influence upon the chemical carcinogenesis. Studies with 3,4-benzopyrene. *Z. Krebsforsch.*, **86**, 85-88
- Schmähl, D., Schmidt, K.G. & Habs, M. (1977) *Syncarcinogenic action of polycyclic hydrocarbons in automobile exhaust gas condensate*. In: Mohr, U., Schmähl, D. & Tomatis, L., eds, *Air Pollution and Cancer in Man (IARC Scientific Publications No. 16)*, Lyon, International Agency for Research on Cancer, pp. 53-59
- Schmähl, D., Habs, M., Lorenz, M. & Wagner, I. (1982) Occurrence of second tumors in man after anticancer drug treatment. *Cancer Treat. Rev.*, **9**, 167-194
- Schmid, W. (1975) The micronucleus test. *Mutat. Res.*, **31**, 9-15
- Schmidt, K.G., Schmähl, D., Misfeld, J. & Timm, J. (1976) Experimental investigations on syncarcinogenesis. 7. Syncarcinogenic effect of polycyclic aromatic hydrocarbons (PAH) after cutaneous administration to mouse skin (Ger.). *Z. Krebsforsch.*, **87**, 93-100
- Schottenfeld, D. & Fraumeni, J.F., Jr, eds (1982) *Cancer Epidemiology and Prevention*, Philadelphia, W.B. Saunders Co.
- Schreiber, G., Urban, J., Zähringer, J., Rutter, W. & Frosch, U. (1971) The secretion of serum proteins and the synthesis of albumin and total protein in regenerating rat liver. *J. biol. Chem.*, **246**, 4531-4538
- Schrenk, D., Schwarz, M., Tennekes, H.A. & Kunz, W. (1981) A novel pathway of nitrosamine metabolism in liver microsomes: denitrosation of nitrosamines by cytochrome P-450. *Adv. exp. Med. Biol.*, **136**, 1157-1164

- Schroeder, F. & Gardiner, J.M. (1984) Membrane lipids and enzymes of cultured high- and low-metastatic B16 melanoma variants. *Cancer Res.*, *44*, 3262-3269
- Schulte-Hermann, R. (1985) Tumor promotion in the liver. *Arch. Toxicol.*, *57*, 147-158
- Schulte-Hermann, R., Timmermann-Trosiener, I. & Schuppler, J. (1982) Response of liver foci in rats to hepatic tumor promoters. *Toxicol. Pathol.*, *10*, 63-70
- Schulte-Hermann, R., Timmermann-Trosiener, I. & Schuppler, J. (1983) Promotion of spontaneous preneoplastic cells in rat liver as a possible explanation of tumor production by non-mutagenic compounds. *Cancer Res.*, *43*, 839-844
- Schulte-Hermann, R., Roome, N., Timmermann-Trosiener, I. & Schuppler, J. (1984a) Immunocytochemical demonstration of a phenobarbital-inducible cytochrome P450 in putative preneoplastic foci of rat liver. *Carcinogenesis*, *5*, 149-153
- Schulte-Hermann, R., Timmermann-Trosiener, I. & Schuppler, J. (1984b) *Aberrant expression of adaptation to phenobarbital may cause selective growth of foci of altered cells in rat liver.* In: Börzsönyi, M., Day, N.E., Lapis, K. & Yamasaki, H., eds, *Models, Mechanisms and Etiology of Tumour Promotion (IARC Scientific Publications No. 56)*, Lyon, International Agency for Research on Cancer, pp. 67-75
- Schulte-Hermann, R., Timmermann-Trosiener, I. & Schuppler, J. (1986) Facilitated expression of adaptive responses to phenobarbital in putative prestages of liver cancer. *Carcinogenesis*, *7*, 1651-1655
- Schuman, R.F., Leberherz, W.B. & Pienta, R.J. (1981) Induction of morphological transformation of hamster embryo cells *in vitro* by diphenylnitrosamine. *Carcinogenesis*, *2*, 679-682
- Schuppler, J., Schulte-Hermann, R., Timmermann-Trosiener, I. & Günzel, P. (1986) Proliferative liver lesions and sex steroids in rats. *Toxicol. Pathol.*, *10*, 132-144
- Schwartz, D.E. (1966) Comparative metabolic studies with Natulan, methylhydrazine and methylamine in rats. *Experientia*, *22*, 212-213
- Schwartz, E.L., Braselton, W.E. & Goodman, J.I. (1981) Factors influencing the binding of *N*-2-fluorenylacetamide to specific regions of the hepatic genome *in vivo* in rats. *J. natl Cancer Inst.*, *66*, 667-672
- Schwarz, M., Appel, K.E., Schrenk, D. & Kunz, W. (1980) Effect of ethanol on microsomal metabolism of dimethylnitrosamine. *J. Cancer Res. clin. Oncol.*, *97*, 233-240
- Schwarz, M., Buchmann, A., Moore, M. & Kunz, W. (1984) *The mechanism of cocarcinogenic action of ethanol in rat liver.* In: Börzsönyi, M., Day, N.E., Lapis, K. & Yamasaki, H., eds, *Models, Mechanisms and Etiology of Tumour Promotion (IARC Scientific Publications No. 56)*, Lyon, International Agency for Research on Cancer, pp. 83-90
- Schweizerisches Bauernsekretariat (1983) *Produktion und Verbrauch von Nahrungsmitteln in der Schweiz 1969/70 bis 1980 (Publikation 141)* [Food Production and consumption in Switzerland 1969/70 to 1980 (Publication 141)], Brugg
- Scicchitano, D., Jones, R.A., Kuzmich, S., Gaffney, B., Lasko, D.D., Essigmann, J.M. & Pegg, A.E. (1986) Repair of oligodeoxynucleotides containing *O*<sup>6</sup>-methylguanine by *O*<sup>6</sup>-alkylguanineDNA-alkyltransferase. *Carcinogenesis*, *7*, 1383-1386
- Sen, N.P. & Donaldson, B. (1978) Improved colorimetric method for determining nitrate and nitrite in foods. *J. Assoc. off. anal. Chem.*, *61*, 1389-1394
- Sen, N.P. & Seaman, S. (1984) *On-line combination of high performance liquid chromatography and total N-nitroso determination apparatus for the determination of N-nitrosamides and other N-nitroso compounds and some recent data on the levels of N-nitrosoproline in foods and beverages.* In: O'Neill, I.K., von Borstel, R.C., Miller, C.T., Long, J. & Bartsch, H., eds, *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer (IARC Scientific Publications No. 57)*, Lyon, International Agency for Research on Cancer, pp. 137-143
- Sen, N.P., Smith, D.C. & Schwinghamer, L. (1969) Formation of *N*-nitrosamines from secondary amines and nitrite in human and animal gastric juice. *Food Cosmet. Toxicol.*, *7*, 301-307

- Sen, N.P., Miles, W.F., Donaldson, B., Panalaks, T. & Iyengar, J.R. (1973) Formation of nitrosamines in a meat curing mixture. *Nature*, *245*, 104-105
- Sen, N.P., Tessier, L. & Seaman, S.W. (1983) Determination of *N*-nitrosoproline and *N*-nitrososarcosine in malt and beer. *J. Agric. Food Chem.*, *31*, 1033-1036
- Sen, N.P., Seaman, S., Clarkson, S., Garrod, F. & Lalonde, P. (1984) *Volatile N-nitrosamines in baby bottle rubber nipples and pacifiers, analysis, occurrence and migration*. In: O'Neill, I.K., von Borstel, R.C., Miller, C.T., Long, J. & Bartsch, H., eds, *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer (IARC Scientific Publications No. 57)*, Lyon, International Agency for Research on Cancer, pp. 51-57
- Sen, N.P., Tessier, L., Seaman, S.W. & Baddoo, P.A. (1985) Volatile and nonvolatile nitrosamines in fish and the effect of deliberate nitrosation under simulated gastric conditions. *J. Agric. Food Chem.*, *33*, 264-268
- Severson, R.F., McDuffie, K.L., Arrendale, R.F., Gwynn, R., Chaplin, J.F. & Johnson, A.W. (1981) Rapid method for the analysis of nicotine alkaloids. *J. Chromatogr.*, *21*, 111-121
- Shank, R.C., Bhamarapravati, N., Gordon, J.E. & Wogan, G.N. (1972) Dietary aflatoxins and human liver cancer. IV. Incidence of primary liver cancer in two municipal populations in Thailand. *Food Cosmet. Toxicol.*, *10*, 171-179
- Shanmugaratnam, K. & Tye, C.Y. (1970) A study of nasopharyngeal cancer among Singapore Chinese with special reference to migrant status and specific community (dialect group). *J. chron. Dis.*, *23*, 433-441
- Shapiro, R. & Klein, R.S. (1966) The deamination of cytidine and cytosine by acidic buffer solutions. Mutagenic implications. *Biochemistry*, *5*, 2358-2362
- Shephard, S.E., Schlatter, C. & Lutz, W.K. (1987) Risk assessment of the formation of carcinogenic *N*-nitroso compounds from dietary precursors in the stomach. *Food Chem. Toxicol.* (in press)
- Shewan, J.M. (1961) *The microbiology of seawater fish*. In: Borgstrom, G., ed., *Fish as Food*, Vol. I, New York, Academic Press, pp. 487-560
- Shieh, J.J., Jenkins, R.K. & Wierbicki, E. (1985) Dosimetry and dose distribution in cesium-137 irradiation unit used at the Eastern Regional Research Center. *Radiat. Phys. Chem.*, *25*, 779-792
- Shimada, A. (1980) A study on dietary habits of populations in Akita and Iwate prefectures with different gastric cancer risks. *Akita J. Med.*, *7*, 153-178
- Shimada, H. & Hayatsu, H. (1985) Mutagenicity arising from near-ultraviolet irradiation of *N*-nitrosomorpholine. *Mutat. Res.*, *143*, 165-168
- Shirai, T., Imaida, K., Fukushima, S., Hasagawa, R., Tatematsu, M. & Ito, N. (1982) Effects of NaCl, Tween 60 and low dose of *N*-ethyl-*N*-nitro-*N*-nitrosoguanidine on gastric carcinogenesis of rat given a single dose MNNG. *Carcinogenesis*, *3*, 1419-1422
- Shuker, D.E.G. & Tannenbaum, S.R. (1983) Determination of nonvolatile *N*-nitroso compounds in biological fluids by liquid chromatography with postcolumn photohydrolysis detection. *Anal. Chem.*, *55*, 2152-2155
- Shuker, D.E.G., Tannenbaum, S.R. & Wishnok, J.S. (1981) *N*-Nitrosobile acid conjugates. I. Synthesis, chemical reactivity and mutagenic activity. *J. org. Chem.*, *46*, 2092-2096
- Shuker, D.E.G., Bailey, E., Gorf, S.M., Lamb, J. & Farmer, P.B. (1984a) Determination of *N*-7- $[^2\text{H}_3]$ methyl guanine in rat urine by gas chromatography-mass spectrometry following administration of trideuteromethylating agents or precursors. *Anal. Biochem.*, *140*, 270-275
- Shuker, D.E.G., Bailey, E. & Farmer, P.B. (1984b) *Methylation of protein and nucleic acids in vivo: use of trideuteromethylating agents or precursors*. In: In: O'Neill, I.K., von Borstel, R.C., Miller, C.T., Long, J. & Bartsch, H., eds, *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer (IARC Scientific Publications No. 57)*, Lyon, International Agency for Research on Cancer, pp. 589-594
- Silinskas, K.C., Zucker, P.F., Labuc, G.E. & Archer, M.C. (1984) Formation of *O*<sup>6</sup>-methylguanine in regenerating rat liver by *N*-nitrosomethylbenzylamine is not sufficient for initiation of preneoplastic foci. *Carcinogenesis*, *5*, 541-542

- Simarak, S., De Jong, U.W., Breslow, N., Dahl, C.J., Ruckphaopunt, K., Scheelings, P. & MacLennan, R. (1977) Cancer of the oral cavity, pharynx/larynx and lung in North Thailand: case-control study and analysis of cigar smoke. *Br. J. Cancer*, 36, 130-140
- Sina, J.F., Bean, C.L., Dysart, G.R., Taylor, V.I. & Bradley, M.O. (1983) Evaluation of the alkaline elution/rat hepatocyte assay as a predictor of carcinogenic/mutagenic potential. *Mutat. Res.*, 113, 357-391
- Singer, B. (1976) All oxygens in nucleic acids react with carcinogenic ethylating agents. *Nature*, 264, 333-339
- Singer, B. (1982) *Mutagenesis from a chemical perspective: nucleic acid reactions, repair, translation and transcription*. In: Lemontt, J.F. & Generoso, W.M., eds, *Molecular and Cellular Mechanisms of Mutagenesis*, New York, Plenum, pp. 1-42
- Singer, B. (1984) Alkylation of the O<sup>6</sup> of guanine is only one of many chemical events that may initiate carcinogenesis. *Cancer Invest.*, 2, 233-238
- Singer, B. (1986) O<sup>6</sup>-Methyldeoxythymidine replacing deoxythymidine in poly [d(A-T)] renders the polymer resistant to the 3' → 5' exonuclease activity of the Klenow and T4 DNA polymerases. *Nucleic Acids Res.*, 14, 6735-6743
- Singer, B. & Kušmirek, J.T. (1982) Chemical mutagenesis. *Ann. Rev. Biochem.*, 52, 655-693
- Singer, B., Spengler, S. & Bodell, W.J. (1981) Tissue-dependent enzyme-mediated repair or removal of O-ethyl pyrimidines and ethyl purines in carcinogen-treated rats. *Carcinogenesis*, 2, 1069-1073
- Singer, B., Sagi, J. & Kušmirek, J.T. (1983) *Escherichia coli* polymerase I can use O<sup>2</sup>-methyldeoxythymidine or O<sup>4</sup>-methyldeoxythymidine in place of deoxythymidine in primed poly (dA-dT) poly (dA-dT) synthesis. *Proc. natl Acad. Sci. USA*, 80, 4584-4588
- Singer, B., Abbott, L.G. & Spengler, S.J. (1984) Assessment of mutagenic efficiency of two carcinogen-modified nucleosides, 1,N<sup>6</sup>-ethenodeoxyadenosine and O<sup>4</sup>-methyldeoxythymidine, using polymerases of varying fidelity. *Carcinogenesis*, 5, 1165-1171
- Singer, B., Spengler, S.J., Fraenkel-Conrat, H. & Kušmirek, J.T. (1986a) O<sup>4</sup>-Methyl-, ethyl-, or isopropyl substituents on thymidine in poly (dA-dT) all lead to transitions upon replication. *Proc. natl Acad. Sci. USA*, 83, 28-32
- Singer, B., Chavez, F. & Spengler, S.J. (1986b) O<sup>4</sup>-Methyl-, ethyl-, and isopropyl deoxythymidine triphosphates as analogues of deoxythymidine triphosphate: kinetics of incorporation by *Escherichia coli* DNA polymerase. I. *Biochemistry*, 26, 1201-1205
- Singer, G.M. & MacIntosh, W.A. (1984) *Urinary metabolites of some alicyclic nitrosamines*. In: O'Neill, I.K., von Borstel, R.C., Miller, C.T., Long, J. & Bartsch, H., eds, *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer (IARC Scientific Publications No. 57)*, Lyon, International Agency for Research on Cancer, pp. 459-463
- Singer, G.M., Chuan, J., Roman, J., Li, M.H. & Lijinsky, W. (1986) Nitrosamines and nitrosamine precursors in foods from Lin-xian, China, a high incidence area for esophageal cancer. *Carcinogenesis*, 7, 733-736
- Skipper, P.L., Tomera, J.F., Wishnok, J.S., Brunengraber, H. & Tannenbaum, S.R. (1983) Pharmacokinetic model for N-nitrosodimethylamine based on Michaelis-Menten constants determined with the isolated perfused rat liver. *Cancer Res.*, 43, 4786-4790
- Smith, P.A.S. (1965) *The Chemistry of Open-Chain Nitrogen Compounds*, New York, W.A. Benjamin
- Smith, P.L.R., Walters, C.L. & Reed, P.I. (1983) Importance of selectivity in the determination of N-nitroso compounds as a group. *Analyst*, 108, 896-898
- Smith, T.A. (1981) Amines in food. *Food Chem.*, 6, 169-200
- Solt, D. & Farber, E. (1976) New principle of the analysis of chemical carcinogenesis. *Nature*, 263, 701-703
- Solt, D.B., Cayama, E., Tsuda, H., Enomoto, K., Lee, G. & Farber, E. (1983) Promotion of liver cancer development by brief exposure to dietary 2-acetylaminofluorene plus partial hepatectomy or carbon tetrachloride. *Cancer Res.*, 43, 188-191

- Song Puju, Shuker, D.E.G., Bishop, W.W., Falchuk, K.R., Tannenbaum, S.R. & Thilly, W.G. (1982) Mutagenicity of *N*-nitrosobile acid conjugates in *S. typhimurium* and diploid human lymphoblasts. *Cancer Res.*, *42*, 2601-2604
- Sosonowski, A., Rajalakshmi, S. & Sarma, D.S.R. (1976) Protection by dimethylsulfoxide of strand breaks in hepatic DNA induced by dimethylnitrosamine. *Chem.-biol. Interactions*, *15*, 101-104
- Sparks, R.E., Mason, N.S., Meier, P.M., Litt, M.H. & Lindan, O. (1971) Removal of uremic waste metabolites from the intestinal tract by encapsulated carbon and oxidized starch. *Trans. Am. Soc. Artif. Intern. Organs*, *17*, 229-238
- Spencer, H. (1985) *Pre-carcinomatous conditions in the lung*. In: Spencer, H., ed., *Pathology of the Lung*, 4th Ed., New York, Pergamon Press, pp. 851-932
- Spiegelhalder, B. (1983) *Isolation and determination of N-nitrosamines and nitrosatable compounds in rubber and plastic commodities after migration into a saliva test solution*. In: Egan, H., Preussmann, R., O'Neill, I.K., Eisenbrand, G., Spiegelhalder, B. & Bartsch, H., eds, *Environmental Carcinogens, Selected Methods of Analysis*, Vol. 6, *N-Nitroso Compounds (IARC Scientific Publications No. 45)*, Lyon, International Agency for Research on Cancer, pp. 265-273
- Spiegelhalder, B. & Preussmann, R. (1982) *Nitrosamines and rubber*. In: Bartsch, H., O'Neill, I.K., Castegnaro, M. & Okada, M., eds, *N-Nitroso Compounds: Occurrence and Biological Effects (IARC Scientific Publications No. 41)*, Lyon, International Agency for Research on Cancer, pp. 231-243
- Spiegelhalder, B. & Preussmann, R. (1983a) Occupational nitrosamine exposure. 1. Rubber and tyre industry. *Carcinogenesis*, *4*, 1147-1152
- Spiegelhalder, B. & Preussmann, R. (1983b) *Analysis of airborne N-nitrosamines: general aspects and sampling strategies*. In: Preussmann, R., O'Neill, I.K., Eisenbrand, G., Spiegelhalder, B. & Bartsch, H., eds, *Environmental Carcinogens. Selected Methods of Analysis*, Vol. 6, *N-Nitroso Compounds (IARC Scientific Publications No. 45)*, Lyon, International Agency for Research on Cancer, pp. 41-53
- Spiegelhalder, B. & Preussmann, R. (1985) In vivo nitrosation of amidopyrene in humans; use of 'ethanol effect' for biological monitoring of N-nitrosodimethylamine in urine. *Carcinogenesis*, *6*, 545-548
- Spiegelhalder, B., Eisenbrand, G. & Preussmann, R. (1976) Influence of dietary nitrate on nitrite content of human saliva: possible relevance to in-vivo formation of *N*-nitroso compounds. *Food Cosmet. Toxicol.*, *14*, 545-548
- Spiegelhalder, B., Eisenbrand, G. & Preussmann, R. (1979) Contamination of beer with trace quantities of *N*-nitrosodimethylamine. *Food Cosmet. Toxicol.*, *17*, 29-31
- Spiegelhalder, B., Eisenbrand, G. & Preussmann, R. (1980a) *Occurrence of volatile nitrosamines in food: a survey of the West German market*. In: Walker, E.A., Gričiūtė, L., Castegnaro, M. & Börzsönyi, M., eds, *N-Nitroso Compounds: Analysis, Formation and Occurrence (IARC Scientific Publications No. 31)*, Lyon, International Agency for Research on Cancer, pp. 467-477
- Spiegelhalder, B., Eisenbrand, G. & Preussmann, R. (1980b) Volatile nitrosamines in food. *Oncology*, *37*, 211-216
- Spiegelhalder, B., Eisenbrand, G. & Preussmann, R. (1982) *Urinary excretion of N-nitrosamines in rats and humans*. In: Bartsch, H., O'Neill, I.K., Castegnaro, M. & Okada, M., eds, *N-Nitroso Compounds: Occurrence and Biological Effects (IARC Scientific Publications No. 41)*, Lyon, International Agency for Research on Cancer, pp. 443-449
- Spiegelhalder, B., Eisenbrand, G. & Preussmann, R. (1983) *Volatile N-nitrosamines in beer and other beverages by direct extraction using a Kieselguhr column*. In: Egan, H., Preussmann, R., O'Neill, I.K., Eisenbrand, G., Spiegelhalder, B. & Bartsch, H., eds, *Environmental Carcinogens. Selected Methods of Analysis*, Vol. 6, *N-Nitroso Compounds (IARC Scientific Publications No. 45)*, Lyon, International Agency for Research on Cancer, pp. 135-142
- Spiegelhalder, B., Hartung, M. & Preussmann, R. (1984) *Biological monitoring in the metal-working industry*. In: O'Neill, I.K., von Borstel, R.C., Müller, C.T., Long, J. & Bartsch, H., eds, *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer (IARC Scientific Publications No. 57)*, Lyon, International Agency for Research on Cancer, pp. 943-946

- Spinelli-Gugger, A.M., Lakritz, L., Wasserman, A.E. & Gates, R.A. (1981) Nitrosamine precursors in the adipose tissue of pork bellies. *J. Food Sci.*, *43*, 1136-1138, 1151
- Srianujata, S., Tangbanleukul, L., Bunyaratvej, S. & Valyasevi, A. (1984) Nitrate and nitrite in saliva and urine of inhabitants of areas of low and high incidence of cholangiocarcinoma in Thailand. In: O'Neill, I.K., von Borstel, R.C., Miller, C.T., Long, J. & Bartsch, H., eds, *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer (IARC Scientific Publications No. 57)*, Lyon, International Agency for Research on Cancer, pp. 921-927
- Staffa, J.A. & Mehlman, M.A. (1979) *Innovations in Cancer Risk Assessments (ED<sub>01</sub> Study)*, Park Forest South, IL, Pathotox Publishers
- Stalsburg, H. & Taktsdal, S. (1971) Stomach cancer following surgery for benign conditions. *Lancet*, *ii*, 1175-1178
- Steele, C.M., Lilies, M. & Ioannides, C. (1985) Inhibition of the mutagenicity of aromatic amines by the plant flavonoid (+)-catechin. *Cancer Res.*, *45*, 3573-3577
- Sterzel, W. (1986) *Metabolism and Cause of the Genotoxic Effect of N-Nitrosoethanolamine (Ger.)*, PhD Thesis, University of Kaiserslautern, Federal Republic of Germany
- Sterzel, W. & Eisenbrand, G. (1986) N-Nitrosodiethanolamine is activated in the rat to an ultimate genotoxic metabolite by sulfotransferase. *J. Cancer Res. clin. Oncol.*, *111*, 20-24
- Sterzel, W., Bedford, P. & Eisenbrand, G. (1985) Automated determination of DNA using the fluorochrome Hoechst 33258. *Anal. Biochem.*, *147*, 462-467
- Stewart, A., Webb, J. & Hewitt, D.A. (1958) A survey of childhood malignancy. *Br. med. J.*, *i*, 1495-1508
- Stewart, B.W. (1981) Generation and persistence of carcinogen-induced repair intermediates in rat liver DNA *in vivo*. *Cancer Res.*, *41*, 3238-3243
- Stewart, B.W., Swann, P.F., Holsman, J.W. & Magee, P.N. (1974) Cellular injury and carcinogenesis. Evidence for alkylation of rat liver nucleic acids *in vivo* by N-nitrosomorpholine. *Z. Krebsforsch.*, *82*, 1-12
- Stewart, B.W., Hristofordidis, C. & Haber, M. (1985) Dose dependent persistence of alkylation-induced single stranded regions in rat liver DNA *in vivo*. *Cancer Lett.*, *28*, 27-33
- Stich, H.F., Ohshima, H., Pignatelli, B., Michelon, J. & Bartsch, H. (1983) Inhibitory effect of betel nut extracts on endogenous nitrosation in humans. *J. natl Cancer Inst.*, *70*, 1047-1050
- Stich, H.F., Hornby, A.P. & Dunn, B.P. (1984a) The effect of dietary factors on nitrosoproline levels in human urine. *Int. J. Cancer*, *33*, 625-628
- Stich, H.F., Dunn, B.P., Pignatelli, B., Ohshima, H. & Bartsch, H. (1984b) *Dietary phenolics and betel nut extracts as modifiers of N-nitrosation in rat and man*. In: O'Neill, I.K., von Borstel, R.C., Miller, C.T., Long, J. & Bartsch, H., eds, *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer (IARC Scientific Publications No. 57)*, Lyon, International Agency for Research on Cancer, pp. 213-222
- Stier, A., Clauss, R., Lücke, A. & Reitz, I. (1982) *Radicals in carcinogenesis by aromatic amines*. In: McBrien, D.C.H.M. & Slater, T.F., eds, *Free Radicals, Lipid Peroxidation and Cancer*, New York, Academic Press, pp. 329-351
- Stjernfeldt, M., Lindsten, J., Berglund, K. & Ludvigsson, J. (1986) Maternal smoking during pregnancy and risk of childhood cancer. *Lancet*, *i*, 1350-1352
- Stockbrugger, R.W., Cotton, P.B., Eugenides, N., Bartholomew, B.A., Hill, M.J. & Walters, C.L. (1982) Intragastric nitrites, nitrosamines and bacterial over-growth during cimetidine treatment. *Gut*, *23*, 1048-1054
- Stoner, G.D., Daniel, F.B., Schenck, K.M., Schut, H.A.J., Goldblatt, P.J. & Sandwisch, D.W. (1982) Metabolism and DNA binding of benzo[a]pyrene in cultured human bladder and bronchus. *Carcinogenesis*, *3*, 195-201
- Stuehr, D.J. & Marletta, M.A. (1985) Mammalian nitrate biosynthesis: mouse macrophages produce nitrite and nitrate in response to *Escherichia coli* lipopolysaccharide. *Proc. natl Acad. Sci. USA*, *82*, 7738-7742

- Sturniolo, G., Carditello, A., Bonavita, G., Bartolotta, M. & Saitta, E. (1983) Risk factors for development of primary cancer in the gastric stump. *Acta chir. scand.*, **149**, 591-596
- Sugimura, T., Matsukura, N. & Sato, S. (1982) *Intestinal metaplasia of the stomach as a precancerous stage*. In: Bartsch, H. & Armstrong, B., eds, *Host Factors in Human Carcinogenesis (IARC Scientific Publications No. 39)*, Lyon, International Agency for Research on Cancer, pp. 516-530
- Sukumar, S., Notario, V., Martin-Zanca, D. & Barbacid, M. (1983) Induction of mammary carcinomas in rats by nitroso-methyl-urea involves malignant activation of Ha-ras-1 locus by single point mutations. *Nature*, **306**, 658-661
- Sumi, Y. & Miyakawa, M. (1981) Comparative studies on the production of stomach tumours following the incubation of several doses of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in germ-free and conventional newborn rats. *Gann*, **72**, 700-704
- Suzuki, E. & Okada, M. (1979) Synthesis of *N*-nitrosamino aldehydes, metabolic intermediates possibly involved in the induction of tumors in rats by *N*-butyl-*N*-( $\omega$ -hydroxyalkyl)nitrosamines. *Chem. Pharm. Bull.*, **27**, 541-544
- Suzuki, E. & Okada, M. (1980) Metabolic fate of *N,N*-dibutylnitrosamine in the rat. *Gann*, **71**, 863-870
- Suzuki, E. & Okada, M. (1985) Metabolic fate of  $\omega$ -1 and  $\omega$ -2 oxidized *N*-nitrosodibutylamines in the rat. *Jpn. J. Cancer Res. (Gann)*, **76**, 173-183
- Suzuki, E., Mochizuki, M., Shibuya, K. & Okada, M. (1983a) *N,N*-Dibutylnitrosamine metabolism by rat liver S9 fraction and oxidation by chemical model systems. *Gann*, **74**, 41-50
- Suzuki, E., Mochizuki, M., Wakabayashi, Y. & Okada, M. (1983b) In vitro metabolic activation of *N,N*-dibutylnitrosamine in mutagenesis. *Gann*, **74**, 51-59
- Suzuki, E., Mochizuki, M., Sekiguchi, N., Osabe, M. & Okada, M. (1985) In vitro metabolism of *N*-nitrodialkylamines. *Jpn. J. Cancer Res. (Gann)*, **76**, 28-36
- Suzuki, E., Mochizuki, M., Suzuki, T. & Okada, M. (1986) Metabolic fate of *N*-nitrodibutylamine in the rat. *Jpn. J. Cancer Res. (Gann)*, **77**, 39-44
- Suzuki, K. & Mitsuoka, T. (1984) *N-Nitrosamine formation by intestinal bacteria*. In: O'Neill, I.K., von Borstel, R.C., Miller, C.T., Long, J. & Bartsch, H., eds, *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer (IARC Scientific Publications No. 57)*, Lyon, International Agency for Research on Cancer, pp. 275-281
- Suzuki, T., Sasaki, K., Takeda, M. & Uchiyama, M. (1984) Some *S*-containing metabolites of tributyl phosphate in the rat. *J. Agric. Food Chem.*, **32**, 1278-1283
- Suzuki, Y., Mutsushi, M. & Ogiu, T. (1984) Morphologic characteristics of thymic lymphomas induced by *N*-nitroso-*N*-propylurea in F344 rats. *J. natl. Cancer Inst.*, **72**, 367-373
- Swann, P.F. (1982) *Metabolism of nitrosamines: observations on the effect of alcohol on nitrosamine metabolism and human cancer*. In: Magee, P.N., ed., *Nitrosamines and Human Cancer (Banbury Report 12)*, Cold Spring Harbor, NY, CSH Press, pp. 53-68
- Swann, P.F. & McLean, A.E.M. (1968) The effect of diet on the toxic and carcinogenic action of dimethylnitrosamine. *Biochem. J.*, **107**, 14-15
- Swann, P.F., Mace, R., Angeles, R.M. & Keefer, L.K. (1983) Deuterium isotope effect on metabolism of *N*-nitrosodimethylamine *in vivo* in rat. *Carcinogenesis*, **4**, 821-825
- Swann, P.F., Coe, A.M. & Mace, R. (1984) Ethanol and dimethylnitrosamine and diethylnitrosamine metabolism and disposition in the rat. Possible relevance to the influence of ethanol on human cancer incidence. *Carcinogenesis*, **5**, 1337-1343
- Swenberg, J.A. & Bedell, M.A. (1982) *Cell-specific DNA alkylation and repair: application of new fluorometric techniques to detect adducts*. In: Magee, P.N., ed., *Indicators of Genotoxic Exposure (Banbury Report 13)*, Cold Spring Harbor, NY, CSH Press, pp. 205-220
- Swenberg, J.A., Koestner, A., Wechsler, W., Brunden, M.N. & Abe, H. (1975) Differential oncogenic effects of methylnitrosourea. *J. natl. Cancer Inst.*, **54**, 89-96

- Swenberg, J.A., Kerns, W.D., Mitchell, R.I., Caralla, E.J. & Pavkov, K.L. (1980) Induction of squamous cell carcinomas of the rat nasal cavity by inhalation exposure to formaldehyde vapor. *Cancer Res.*, *40*, 3398-3402
- Swenberg, J.A., Dyroff, M.C., Bedell, M.A., Popp, J.A., Huh, N., Kirstein, U. & Rajewsky, M.F. (1984) *O*<sup>4</sup>-Ethyldeoxythymidine, but not *O*<sup>6</sup>-ethyldeoxyguanosine, accumulates in hepatocyte DNA of rats exposed continuously to diethylnitrosamine. *Proc. natl Acad. Sci. USA*, *81*, 1692-1695
- Swenson, D.H., Farmer, P.B. & Lawley, P.D. (1976) Identification of the methyl phosphotriester of thymidylyl(3' → 5')thymidine as a product from reaction of DNA with the carcinogen *N*-methyl-*N*-nitrosourea. *Chem.-biol. Interactions*, *15*, 91-100
- Tacchi, A.M., Schmähl, D. & Habs, M. (1984) Delay of bladder cancer induction in rats treated with *N*-nitroso-*N*-butyl-*N*-(4-hydroxybutyl)amine by administration of sodium 2-mercaptoethanesulfonate (mesna). *Cancer Lett.*, *22*, 89-94
- Tahira, T., Tsuda, M., Wakabayashi, K., Nagao, M. & Sugimura, T. (1984) Kinetics of nitrosation of thioproline, the precursor of a major nitroso compound in human urine, and its role as a nitrite scavenger. *Gann*, *75*, 889-894
- Takano, S., Akagi, M. & Bryan, G.T. (1984) Stimulation of ornithine decarboxylase activity and DNA synthesis by phorbol esters or bile acids in rat colon. *Gann*, *75*, 29-35
- Takeda, Y. & Kanaya, H. (1982) A screening procedure for the formation of nitroso derivatives and mutagens by drug-nitrite interaction. *Chem. Pharm. Bull.*, *30*, 3399-3404
- Tannenbaum, S.R. & Correa, P. (1985) Nitrate and gastric cancer risks. *Nature*, *317*, 675-676
- Tannenbaum, S.R., Sinskey, A.J., Weisman, M. & Bishop, W. (1974) Nitrite in human saliva. Its possible relationship to nitrosamine formation. *J. natl Cancer Inst.*, *53*, 79-84
- Tannenbaum, S.R., Archer, M.C., Wishnok, J.S. & Bishop, W.W. (1978) Nitrosamine formation in human saliva. *J. natl Cancer Inst.*, *60*, 251-253
- Tannenbaum, S.R., Moran, D., Falchuk, K.R., Correa, P. & Cuello, C. (1981) Nitrite stability and nitrosation potential in human gastric juice. *Cancer Lett.*, *14*, 131-136
- Tannenbaum, S.R., Bishop, W., Yu, M.C. & Henderson, B.E. (1985) Attempts to isolate *N*-nitroso compounds from Chinese-style salted fish. *Natl Cancer Inst. Monogr.*, *69*, 209-211
- Tata, J.R. & Baker, B. (1978) Enzymatic fractionation of nuclei. Polynucleases and RNA polymerase II as endogenous transcriptional complexes. *J. mol. Biol.*, *118*, 249-272
- Taylor, H.W. & Lijinsky, W. (1975) Tumor induction in rats by feeding aminopyrine or oxytetracycline with nitrite. *Int. J. Cancer*, *16*, 211-215
- Terracini, B., Magee, P.N. & Barnes, J.M. (1967) Hepatic pathology in rats on low dietary levels of dimethylnitrosamines. *Br. J. Cancer*, *21*, 559-565
- Thamavit, W., Bhamarapravati, N., Sahapong, S., Vajarasthira, S. & Angsubhakorn, S. (1978) Effect of dimethylnitrosamine on induction of cholangiocarcinoma in *Opithorchis viverrini*-infected Syrian golden hamsters. *Cancer Res.*, *38*, 4634-4639
- de-Thé, G. (1982) *Epidemiology of the Epstein-Barr virus and associated diseases*. In: Roizman, B., ed., *The Herpesviruses*, Vol. A1, New York, Plenum, pp. 25-103
- The Chemical Daily Co. (1984) *The Specialty Chemicals Handbook*, Tokyo, pp. 309-316
- Thompson, H.C., Jr, Billedeau, S.M., Miller, B.J., Hansen, E.B., Jr & Freeman, J.P. (1984). Determination of *N*-nitrosamines and *N*-nitrosamine precursors in rubber nipples from baby pacifiers by gas chromatography thermal energy analysis. *J. Toxicol. environ. Health*, *13*, 615-632
- Tjälve, H. & Löfberg, B. (1983) Extrahepatic metabolism of carbon tetrachloride in rats. *Chem.-biol. Interactions*, *46*, 299-316
- Tjälve, H., Castonguay, A. & Rivenson, A. (1985) Microautoradiographic localization of bound metabolites in the nasal cavities of F344 rats treated with the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. *J. natl Cancer Inst.*, *74*, 185-189



- Tokunaga, T. (1970) Trimethylamine oxide and its decomposition in the dark muscle of fish. I and II. *Bull. Jpn Soc. Sci. Fish.*, *36*, 502-509, 510-515
- Tomera, J.F., Skipper, P.L., Wishnok, J.S., Tannenbaum, S.R. & Brunengrapur, H. (1984) Inhibition of *N*-nitrosodimethylamine metabolism by EtOH and other inhibitors in the isolated perfused liver. *Carcinogenesis*, *5*, 113-116
- Tong, W.P., Kohn, K.W. & Ludlum, D.B. (1982) Modifications of DNA by different haloethylnitrosoureas. *Cancer Res.*, *42*, 4460-4464
- Topal, M.D., Eadie, J.S. & Conrad, M. (1986) *O*<sup>6</sup>-Methylguanine mutation and repair is nonuniform. *J. biol. Chem.*, *261*, 9879-9885
- Topley, M. (1973) *Cultural and social factors relating to Chinese infant feeding and weaning*. In: Field, C.E. & Baber, F.M., eds, *Growing Up in Hong Kong*, Hong Kong, Hong Kong University Press, pp. 56-65
- Tricker, A.R., Perkins, M.J., Massey, R.C. & McWeeny, D.J. (1985) *N*-Nitrosopyrrolidine formation in bacon. *Food Addit. Contam.*, *2*, 247-252
- Tsuda, M., Hirayama, T. & Sugimura, T. (1983) Presence of *N*-nitroso-*L*-thiopropine and *N*-nitroso-*L*-methylthiopropines in human urine as major *N*-nitroso compounds. *Gann*, *74*, 331-333
- Tsuda, M., Kakizoe, T., Hirayama, T. & Sugimura, T. (1984) *New type of N-nitrosamino acids, N-nitroso-L-thiopropine and N-nitroso-L-methylthiopropines, found in human urine as major N-nitroso compounds*. In: O'Neill, I.K., von Borstel, R.C., Miller, C.T., Long, J. & Bartsch, H., eds, *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer (IARC Scientific Publications No. 57)*, Lyon, International Agency for Research on Cancer, pp. 223-230
- Tsuda, M., Niitsuma, J., Sato, S., Hirayama, T., Kakizoe, T. & Sugimura, T. (1986) Increase in the levels of *N*-nitrosopropine, *N*-nitrosothiopropine and *N*-nitroso-2-methylthiopropine in human urine by cigarette smoking. *Cancer Lett.*, *30*, 117-124
- Tsujihara, K., Ozeki, M., Morikawa, T., Tuya, N., Miyazaki, M., Kawamori, M. & Arai, Y. (1981) A new class of nitrosoureas. II. Synthesis and antitumor activity of 1-(2-chloroethyl)-3,3-disubstituted-1-nitrosoureas having a glucopyranosyl, mannopyranosyl or galactopyranosyl moiety. *Chem. Pharm. Bull.*, *29*, 3262-3273
- Tsung-Hsieu, C., Yu-Chung, L., Kwang-Yung, L., Chang-Hsieu, S. & Yee-Ping, C. (1983) Cocarcinogenic action of aspirin on gastric tumors induced by MNNG in rats. *J. natl Cancer Inst.*, *70*, 1067-1075
- Tu, S.-I., Byler, D.M. & Cavanaugh, J.R. (1984) Nitrite inhibition of acyltransfer by coenzyme A via the formation of an *S*-nitrosothiol derivative. *J. Sci. Food Agric.*, *32*, 1057-1060
- Tu, Y.Y. & Yang, C.S. (1983) High-affinity nitrosamine dealkylase system in rat liver microsomes and its induction by fasting. *Cancer Res.*, *43*, 623-629
- Tu, Y.Y. & Yang, C.S. (1985) Demethylation and denitrosation of nitrosamines by cytochrome P-450 isozymes. *Arch. Biochem. Biophys.*, *242*, 32-40
- Tu, Y.Y., Sonnenberg, J., Lewis, K.F. & Yang, C.S. (1981) Pyrazole-induced cytochrome P-450 in rat liver microsomes: an isozyme with high affinity for dimethylnitrosamine. *Biochem. biophys. Res. Commun.*, *103*, 905-912
- Tu, Y.Y., Peng, R., Chang, Z.-F. & Yang, C.S. (1983) Induction of a high affinity nitrosamine demethylase in rat liver microsomes by acetone and isopropanol. *Chem.-biol. Interactions*, *44*, 247-260
- Tuyns, A.J., Castegnaro, M., Toussaint, G., Walker, E.A., Gričiute, L., Le Talaer, J.Y., Loquet, C., Guérain, J. & Drilleau, J.F. (1980) Research on etiological factors for oesophageal cancer in the west of France (Fr.). *Bull. Cancer*, *67*, 2-28
- Tuyns, A.J., Riboli, E. & Doornbos, G. (1985) *Nutrition and cancer of the esophagus*. In: Joossens, J.V., Hill, M.J. & Geboers, J., eds, *Diet and Human Carcinogenesis*, Amsterdam, Excerpta Medica, pp. 71-79

- Ueno, Y. (1985) The toxicology of mycotoxins. *CRC crit. Rev. Toxicol.*, *14*, 99-132
- Ueno, Y. & Matsumoto, H. (1975) Inactivation of some thiol enzymes by trichothecene mycotoxins from *Fusarium* species. *Chem. Pharm. Bull.*, *23*, 2439-2442
- Ullrich, V., Weber, P. & Wollenberg, P. (1975) Tetrahydrofuran — an inhibitor for ethanol-induced liver microsomal cytochrome P-450. *Biochem. biophys. Res. Commun.*, *64*, 808-813
- Umano, K., Shibamoto, T., Fernando, S.Y. & Wei, C.I. (1984) Mutagenicity of 2-hydroxyalkyl-N-nitrosothiazolidines. *Food Chem. Toxicol.*, *22*, 253-259
- Umbenhauer, D., Wild, C.P., Montesano, R., Saffhill, R., Boyle, J.M., Huh, N., Kirstein, U., Thomale, J., Rajewsky, M.F. & Lu, S.H. (1985) O<sup>6</sup>-Methyldeoxyguanosine in oesophageal DNA among individuals at high risk of oesophageal cancer. *Int. J. Cancer*, *36*, 661-665
- Underwood, B. & Lijinsky, W. (1982) Comparative metabolism of 2,6-dimethylmorpholine in rats, hamsters, and guinea pigs. *Cancer Res.*, *42*, 54-58
- US Department of Agriculture, Agriculture Research Service (1972) *Composition of Foods: Raw, Processed, Prepared*, Washington DC, US Government Printing Office
- US Department of Health and Human Services (1986) *The Health Consequences of Using Smokeless Tobacco (NIH Publication 86-2874)*, Bethesda, MD, US Government Printing Office
- Vanderslice, R.R., Boyd, J.A., Eling, T.E. & Philpot, R.M. (1985) The cytochrome P-450 monooxygenase system of rabbit bladder mucosa: enzyme components and isozyme 5-dependent metabolism of 2-aminofluorene. *Cancer Res.*, *45*, 5851-5858
- Van Stee, E.W., Sloane, R.A., Simmons, J.E. & Brunnemann, K.D. (1983) In-vivo formation of N-nitrosomorpholine in CD-1 mice exposed by inhalation to nitrogen dioxide and by gavage to morpholine. *J. natl Cancer Inst.*, *70*, 375-379
- Van Steensel-Moll, H., Valkenburg, H.A., Vandenbroucke, J.P. & Van Zanen, G.E. (1985) Are maternal fertility problems related to childhood leukaemia? *Int. J. Epidemiol.*, *14*, 555-559
- Varleg, H. (1967) *Practical Clinical Biochemistry*, London, Medical Books & Interscience Books, Inc., pp. 197-198
- Voigt, J.M., Guengerich, F.P. & Baron, J. (1985) Localization of a cytochrome P-450 isozyme (cytochrome P-450 PB-B) and NADPH-cytochrome P-450 reductase in rat nasal mucosa. *Cancer Lett.*, *27*, 241-247
- Vousden, K.H., Bos, J.L., Marshall, C.J. & Phillips, D.H. (1986) Mutations activating human c-Ha-ras-1 protooncogene (HRAS1) induced by chemical carcinogens and depurination. *Proc. natl Acad. Sci. USA*, *83*, 1222-1226
- Wagner, D.A. & Tannenbaum, S.R. (1982) *Enhancement of nitrate biosynthesis by E. coli lipopolysaccharide*. In: Magee, P.N., ed., *Nitrosamines and Human Cancer (Banbury Report 12)*, Cold Spring Harbor, NY, CSH Press, pp. 437-441
- Wagner, D.A., Schultz, D.S., Deen, W.M., Young, V.R. & Tannenbaum, S.R. (1983a) Metabolic fate of an oral dose of <sup>15</sup>N-labeled nitrate in humans: effect of diet supplementation with ascorbic acid. *Cancer Res.*, *43*, 1921-1925
- Wagner, D.A., Young, V.R. & Tannenbaum, S.R. (1983b) Mammalian nitrate biosynthesis: incorporation of [<sup>15</sup>N]-ammonium into nitrate is enhanced by endotoxin treatment. *Proc. natl Acad. Sci. USA*, *80*, 4518-4521
- Wagner, D.A., Shuker, D.E.G., Bilmazes, C., Obiedzinski, M., Young, V.R. & Tannenbaum, S.R. (1984a) *Modulation of endogenous synthesis of N-nitrosamino acids in humans*. In: O'Neill, I.K., von Borstel, R.C., Miller, C.T., Long, J. & Bartsch, H., eds, *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer (IARC Scientific Publications No. 57)*, Lyon, International Agency for Research on Cancer, pp. 223-229
- Wagner, D.A., Young, V.R. & Tannenbaum, S.R. (1984b) *Mammalian nitrate biochemistry: metabolism and endogenous synthesis*. In: O'Neill, I.K., von Borstel, R.C., Miller, C.T., Long, J. & Bartsch, H., eds, *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer (IARC Scientific Publications No. 57)*, Lyon, International Agency for Research on Cancer, pp. 247-253

- Wagner, D.A., Shuker, D.E.G., Bilmazes, C., Obiedzinski, M., Baker, I., Young, V.R. & Tannenbaum, S.R. (1985) Effect of vitamins C and E on endogenous synthesis of *N*-nitrosoamino acids in humans: precursor-product studies with <sup>15</sup>N-nitrate. *Cancer Res.*, **45**, 6519-6522
- Wagner, G., Habs, M. & Schmähl, D. (1982) Influence of daily subcutaneous administration of 6-methylprednisolone and oral application of 30% saline solution on tumorigenesis induced in mouse stomach by single application of 7,12-dimethylbenz(a)anthracene. *J. Cancer Res. clin. Oncol.*, **103**, 207-209
- Wakabayashi, K., Nagao, M., Kawachi, T. & Sugimura, T. (1982) *Mechanism of appearance of mutagenicity of N-nitrosodiphenylamine with norharman*. In: Bartsch, H., O'Neill, I.K., Castegnaro, M. & Okada, M., eds, *N-Nitroso Compounds: Occurrence and Biological Effects (IARC Scientific Publications No. 41)*, Lyon, International Agency for Research on Cancer, pp. 695-707
- Wakabayashi, K., Ochiai, M., Saito, H., Tsuda, M., Suwa, Y., Nagao, M. & Sugimura, T. (1983) Presence of 1-methyl-1,2,3,4-tetrahydro- $\beta$ -carboline-3-carboxylic acid, a precursor of a mutagenic nitroso compound, in soy sauce. *Proc. natl Acad. Sci. USA*, **80**, 2912-2916
- Wakabayashi, K., Nagao, M., Ochiai, M., Tsuda, M., Yamaizumi, Z., Saito, H. & Sugimura, T. (1984) *Presence of 1-methyl-1,2,3,4-tetrahydro- $\beta$ -carboline-3-carboxylic acids and tyramine as precursors of mutagens in soya sauce after nitrite treatment*. In: O'Neill, I.K., von Borstel, R.C., Miller, C.T., Long, J. & Bartsch, H., eds, *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer (IARC Scientific Publications No. 57)*, Lyon, International Agency for Research on Cancer, pp. 17-24
- Wakabayashi, K., Nagao, M., Ochiai, M., Tahira, T., Yamaizumi, Z. & Sugimura, T. (1985a) A mutagen precursor in Chinese cabbage, indole-3-acetonitrile, which becomes mutagenic on nitrite treatment. *Mutat. Res.*, **143**, 17-21
- Wakabayashi, K., Nagao, M., Chung, T.H., Yin, M., Karai, I., Ochiai, M., Tahira, T. & Sugimura, T. (1985b) Appearance of direct-acting mutagenicity of various foodstuffs produced in Japan and Southeast Asia on nitrite treatment. *Mutat. Res.*, **158**, 119-124
- Wakabayashi, K., Nagao, M., Tahira, T., Saito, H., Katayama, M., Marumo, S. & Sugimura, T. (1985c) 1-Nitrosoindole-3-acetonitrile, a mutagen produced by nitrite treatment of indole-3-acetonitrile. *Proc. Jpn Acad. Ser. B*, **61**, 190-192
- Wakabayashi, K., Nagao, M., Tahira, T., Yamaizumi, Z., Katayama, M., Marumo, S. & Sugimura, T. (1986) 4-Methoxyindole derivatives as nitrosable precursors of mutagens in Chinese cabbage. *Mutagenesis* (in press)
- Wakata, A., Oka, N., Hiramoto, K., Yoshioka, A., Negishi, K., Wataya, Y. & Hayatsu, H. (1985) DNA strand cleavage *in vitro* by 3-hydroxyamino-1-methyl-5H-pyrido[4,3-*b*]indole, a direct-acting mutagen formed in the metabolism of carcinogenic 3-amino-1-methyl-5H-pyrido[4,3-*b*]indole. *Cancer Res.*, **45**, 5867-5871
- Waldren, C., Correll, L., Sognier, M.A. & Puck, T.T. (1986) Measurement of low levels of x-ray mutagenesis in relation to human disease. *Proc. natl Acad. Sci. USA*, **83**, 4839-4844
- Walker, E.A. & Castegnaro, M. (1980) Response of nitramines in the thermal analyser. *J. Chromatogr.*, **187**, 229-231
- Walles, S. & Ehrenberg, L. (1968) Effects of  $\beta$ -hydroxyethylation and  $\beta$ -methoxyethylation on DNA *in vitro*. *Acta chem. Scand.*, **22**, 2727-2729
- Walsh, C. (1979) *Enzymatic Reaction Mechanisms*, San Francisco, Freeman, pp. 108-123
- Walters, C.L., Downes, M.J., Edwards, M.W. & Smith, P.L.R. (1978) Determination of a non-volatile *N*-nitrosamine on a food matrix. *Analyst*, **103**, 1127-1133
- Walters, C.L., Carr, F.P.A., Dyke, C.S., Saxby, M.J., Smith, P.L.R. & Walker, R. (1979) Nitrite sources and nitrosamine formation *in vitro* and *in vivo*. *Food Chem. Toxicol.*, **17**, 473-479
- Walters, C.L., Hart, R.J., Keefer, L.K. & Newberne, P.M. (1980) *The sequential determination of nitrite, N-nitroso compounds and nitrate and its application*. In: Walker, E.A., Griciūtė, L., Castegnaro, M. & Börzsönyi, M., eds, *N-Nitroso Compounds: Analysis, Formation and Occurrence (IARC Scientific Publications No. 31)*, Lyon, International Agency for Research on Cancer, pp. 389-399

- Walters, C.L., Smith, P.L.R., Reed, P.I., Haines, K. & House, F.R. (1982) N-Nitroso compounds in gastric juice and their relationship to gastroduodenal disease. In: Bartsch, H., O'Neill, I.K., Castegnaro, M. & Okada, M., eds, *N-Nitroso Compounds: Occurrence and Biological Effects (IARC Scientific Publications No. 41)*, Lyon, International Agency for Research on Cancer, pp. 345-352
- Walters, C.L., Hart, R.J. & Smith, P.L.R. (1983) Analysis of total N-nitroso compounds as a group by denitrosation to nitric oxide, with detection using a chemiluminescence analyser. In: Preussmann, R., O'Neill, I.K., Eisenbrand, G., Spiegelhalder, B. & Bartsch, H., eds, *Environmental Carcinogens. Selected Methods of Analysis*, Vol. 6, N-Nitroso Compounds (IARC Scientific Publications No. 45), Lyon, International Agency for Research on Cancer, pp. 295-308
- Walters, C.L., Smith, P.L.R. & Reed, P.I. (1984) Pitfalls to avoid in determining N-nitroso compounds as a group. In: O'Neill, I.K., von Borstel, R.C., Miller, C.T., Long, J. & Bartsch, H., eds, *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer (IARC Scientific Publications No. 57)*, Lyon, International Agency for Research on Cancer, pp. 113-119
- Walters, C.L., Gillatt, P.N., Palmer, R.C. & Smith, P.L.R. (1986) A rapid method for the determination of nitrate and nitrite by chemiluminescence. *Food Addit. Contam.* (in press)
- Wang, G.H., Zhang, W.X. & Chani, W.G. (1980) The identification of natural Roussin red methyl ester. *Acta chem. sin.*, 38, 95-102
- Wang, M.Y., Lu, S.H., Ji, C., Wang, Y.L. & Jien, C.L. (1983) Determination of nitroso compound (Roussin red) and their precursors in pickled vegetables from Linxian. *Res. Cancer Prev. Treat.*, 10, 12-15
- Wang, T.V. & Cerutti, P. (1980) Spontaneous reactions of AFB<sub>1</sub> modified deoxyribonucleic acid in vitro. *Biochemistry*, 19, 1692-1698
- Wang, Y.L., Lu, S.H. & Li, M.H. (1979) Determination of nitrate and nitrite in well water from Yaocun commune, Linxian county, Henan province. *Chin. J. Oncol.*, 1, 201-205
- Ward, E.J., Haber, M., Norris, M.D. & Stewart, B.W. (1985) Single-strand specific degradation of DNA during isolation of rat liver nuclei. *Biochemistry*, 24, 5803-5809
- Waterhouse, J., Muir, C., Shanmugaratnam, K. & Powell J., eds (1982) *Cancer Incidence in Five Continents, Vol. IV (IARC Scientific Publications No. 42)*, Lyon, International Agency for Research on Cancer
- Watt, P.C.H., Patterson, C.C. & Kennedy, T.L. (1984) Late mortality after vagotomy and drainage for duodenal ulcer. *Br. med. J.*, 288, 1335-1338
- Wattenberg, L.W. & Sparnins, V.L. (1979) Inhibitory effects of butylated hydroxyanisole on methylazoxymethanol acetate-induced neoplasia of the large intestine and on nicotinamide adenine dinucleotide-dependent alcohol dehydrogenase activity in mice. *J. natl Cancer Inst.*, 63, 219-222
- Waydhas, C., Weigl, K. & Sies, H. (1978) The disposition of formaldehyde and formate arising from drug N-demethylations dependent on cytochrome P-450 in hepatocytes and in perfused rat liver. *Eur. J. Biochem.*, 89, 143-147
- Wayss, K., Bannasch, P., Mattern, J. & Volm, M. (1979) Vascular liver tumors induced in *Mastomys (Praomys) natalensis* by single or twofold administration of dimethylnitrosamine. *J. natl Cancer Inst.*, 62, 1199-1207
- Weiner, J.P., Ralph, D.J. & Taylor, L. (1975) Nitrate and nitrite in brewery fermentation. In: *European Brewery Convention, Proceedings of 15th Congress, Nice 1975*, Amsterdam, Elsevier, pp. 565-579
- Weinkam, R.J. & Lin, H.S. (1982) Chloroethylnitrosourea cancer chemotherapeutic agents. *Adv. Pharmacol. Chemother.*, 19, 1-33
- Weisburger, J.H. & Raineri, R. (1975) Dietary factors and the etiology of gastric cancer. *Cancer Res.*, 35, 3469-3474

- Weisburger, J.H., Marquardt, H., Hirota, H., Mori, N. & Williams, G.M. (1980) Induction of cancer of the glandular stomach in rats by extract of nitrite treated fish. *J. natl Cancer Inst.*, *64*, 163-167
- Weissmann, B., Bromberg, P.A. & Gutman, A.B. (1957a) The purine bases of human urine. I. Separation and identification. *J. biol. Chem.*, *224*, 407-422
- Weissmann, B., Bromberg, P.A. & Gutman, A.B. (1957b) The purine bases of human urine. II. Serum quantitative estimation and isotope incorporation. *J. biol. Chem.*, *224*, 423-434
- Wenke, G. & Hoffmann, D. (1983) A study of betel quid carcinogenesis. On the in-vitro *N*-nitrosation of arecoline. *Carcinogenesis*, *4*, 169-172
- Wenke, G., Brunneemann, K.D. & Hoffmann, D. (1984a) A study of betel quid carcinogenesis. IV. Analysis of the saliva of chewers. A preliminary report. *J. Cancer Res. clin. Oncol.*, *108*, 110-113
- Wenke, G., Rivenson, A., Brunneemann, K.D. & Hoffmann, D. (1984b) *A study of betel quid carcinogenesis. 2. On the formation of N-nitrosamines during betel quid chewing.* In: O'Neill, I.K., von Borstel, R.C., Miller, C.T., Long, J. & Bartsch, H., eds, *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer (IARC Scientific Publications No. 57)*, Lyon, International Agency for Research on Cancer, pp. 859-866
- Wenke, G., Rivenson, A. & Hoffmann, D. (1984c) A study of betel quid carcinogenesis. 3. 3-(Methylnitrosamino)propionitrile, a powerful carcinogen in F 344 rats. *Carcinogenesis*, *5*, 1137-1140
- Werringloer, J., Kawano, S., Chacos, N. & Estabrook, R. W. (1979) The interaction of divalent copper and the microsomal electron transport system. *J. biol. Chem.*, *254*, 11839-11846
- Wheeler, G.P., Bowdon, B.J. & Struck, R.F. (1975) Carbamoylation of aminoacids, peptides and proteins by nitrosoureas. *Cancer Res.*, *35*, 2974-2984
- WHO (1978) Definition of leukoplakia and related lesions: an aid to studies on oral precancer. *Oral Surg.*, *46*, 518-539
- Wiessler, M. & Braun, H. (1980)  $\alpha$ -D-Glucuronides of 1-(*N*-alkyl-*N*-nitrosamino)alkyl alcohols. *Angew. Chem. int. Ed.*, *19*, 400-401
- Wiessler, M., Rosnagel, G. & Rugewitz-Blackholm, B. (1984) *Conjugates of N-nitroso-tert-butylmethylamine in urine.* In: O'Neill, I.K., von Borstel, R.C., Miller, C.T., Long, J. & Bartsch, H., eds, *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer (IARC Scientific Publications No. 57)*, Lyon, International Agency for Research on Cancer, pp. 465-471
- Wild, C.P., Smart, G., Saffhill, R. & Boyle, J.M. (1983) Radioimmunoassay of *O*<sup>6</sup>-methyldeoxyguanosine in DNA of cells alkylated *in vitro* and *in vivo*. *Carcinogenesis*, *12*, 1605-1609
- Wild, C.P., Umbenhauer, D., Chapot, B. & Montesano, R. (1986) Monitoring of individual human exposure to aflatoxins (AF) and *N*-nitrosamines (NNO) by immunoassays. *J. Cell Biochem.*, *30*, 171-179
- Willey, J.C., Saladino, A.J., Lechner, J.F. & Harris, C.C. (1984) Acute effects of 12-O-tetradecanoylphorbol-13-acetate, teleocidin, or 2,3,7,8-tetrachlorodibenzo-p-dioxin on cultured normal human bronchial epithelial cells. *Carcinogenesis*, *5*, 209-215
- Winn, D.M., Blot, W.J., Shy, C.M., Pickle, L.M., Toledo, A. & Fraumeni, J.F., Jr (1981) Snuff dipping and oral cancer among women in the southern United States. *New Engl. J. Med.*, *304*, 745-749
- Wishnok, J.S., Snow, K. & Woolworth, V. (1982) *Passage of nitrosamines through animal membranes.* In: Bartsch, H., O'Neill, I.K., Castegnaro, M. & Okada, M., eds, *N-Nitroso Compounds: Occurrence and Biological Effects (IARC Scientific Publications No. 41)*, Lyon, International Agency for Research on Cancer, pp. 435-442
- Wood, A.W., Huang, M.T., Chang, R.L., Newmark, H.L., Lehr, R.E., Yagi, H., Sayer, J.M., Jerina, D.M. & Conney, A.H. (1982) Inhibition of the mutagenicity of bay-region diol epoxides of polycyclic aromatic hydrocarbons by naturally occurring plant phenols: exceptional activity of ellagic acid. *Proc. natl Acad. Sci. USA*, *79*, 5513-5517

- Woodhead, A.D., Merry, B.J., Cao, E., Holehan, A.M., Grist, E. & Carlson, C. (1985) Levels of O<sup>6</sup>-methylguanine acceptor protein in tissues of rats and their relationship to carcinogenicity and aging. *J. natl Cancer Inst.*, *75*, 1141-1145
- Worth, R.M. & Valentine, R. (1967) *Nasopharyngeal carcinoma in New South Wales, Australia*. In: Muir, C. & Shanmugaratnam, K., eds, *Cancer of the Nasopharynx (UICC Monograph Series No. 1)*, Copenhagen, Munksgaard, pp. 73-76
- Wynder, E.L. & Hoffmann, D. (1979) Tobacco and health, a societal challenge. *New Engl. J. Med.*, *300*, 894-903
- Wynder, E.L., Onderdonk, J. & Mantel, N. (1963) An epidemiological investigation of cancer of the bladder. *Cancer*, *16*, 1388-1407
- Wynder, E.L., Goodman, M.T. & Hoffmann, D. (1985) *Lung cancer etiology: challenges of the future*. In: Mass, M.J., Kaufman, D.G., Siegfried, J.M., Steele, V.N. & Nesnow, S., eds, *Carcinogenesis, a Comprehensive Survey*, Vol. 8, New York, Raven Press, pp. 39-62
- Yahagi, T., Nagao, M., Seino, Y., Matsushima, T., Sugimura, T. & Okada, M. (1977) Mutagenicities of N-nitrosamines on *Salmonella*. *Mutat. Res.*, *48*, 121-130
- Yamamoto, S., Wakabayashi, S. & Makita, M. (1980) Gas-liquid chromatographic determination of tyramine in fermented food products. *J. Agric. Food Chem.*, *28*, 790-793
- Yamaoka, K., Tanigawara, Y., Nakagawa, T. & Uno, T. (1981) A pharmacokinetic analysis program (MULTI) for microcomputer. *J. Pharm. Dyn.*, *4*, 879-885
- Yang, C.S. (1980) Research on esophageal cancer: a review. *Cancer Res.*, *40*, 2633-2644
- Yang, C.S. (1982) *Nitrosamines and other etiological factors in the esophageal cancer in Northern China*. In: Magee, P.N., ed., *Nitrosamines and Human Cancer (Banbury Report 12)*, Cold Spring Harbor, NY, CSH Press, pp. 487-501
- Yang, C.S., Strickhart, F.S. & Woo, G.K. (1974) Inhibition of the mono-oxygenase system by butylated hydroxyanisole and butylated hydroxytoluene. *Life Sci.*, *15*, 1497-1505
- Yang, C.S., Miao, J., Yang, W., Huang, M., Wang, T., Xue, H., You, S., Lu, J. & Wu, J. (1982) Diet and vitamin nutrition of the high esophageal cancer risk population in Lin-xian, China. *Nutr. Cancer*, *4*, 154-164
- Yang, C.S., Tu, Y.Y., Hong, J. & Patten, C. (1984) *Metabolism of nitrosamines by cytochrome P-450 isozymes*. In: O'Neill, I.K., von Borstel, R.C., Miller, C.T., Long, J. & Bartsch, H., eds, *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer (IARC Scientific Publications No. 57)*, Lyon, International Agency for Research on Cancer, pp. 423-428
- Yang, C.S., Koop, D.R., Wang, T. & Coon, M.J. (1985a) Immunochemical studies on the metabolism of nitrosamines by ethanol-inducible cytochrome P-450. *Biochem. biophys. Res. Commun.*, *128*, 1007-1013
- Yang, C.S., Tu, Y.Y., Koop, D.R. & Coon, M.J. (1985b) Metabolism of nitrosamines by purified rabbit liver cytochrome P-450 isozymes. *Cancer Res.*, *45*, 1140-1145
- Yang, D., Tannenbaum, S.R., Büchi, G. & Lee, G.C.M. (1984) 4-Chloro-6-methoxyindole is the precursor of a potent mutagen (4-chloro-6-methoxy-2-hydroxy-1-nitroso-indolin-3-one oxime) that forms during nitrosation of the fava bean (*Vicia faba*). *Carcinogenesis*, *5*, 1219-1224
- Yano, K., Katayama, H. & Takemoto, K. (1984) Interrelationship between tumorigenicity and the chemical nature of N-methyl-N'-aryl-N-nitrosoureas. *Cancer Res.*, *44*, 1027-1030
- Yano, K., Sonoda, M. & Sakagishi, Y. (1985) Dynamic changes of the chicken erythrocyte membranes induced by N-methyl-N'-aryl-N-nitrosoureas and their tumorigenicity. *Cancer Lett.*, *26*, 261-268
- Yarosh, D.B. (1985) The role of O<sup>6</sup>-methylguanine-DNA methyltransferase in cell survival, mutagenesis and carcinogenesis. *Mutat. Res.*, *145*, 1-16
- Yarosh, D.B., Fornace, A.J. & Day, R.S. (1985) Human O<sup>6</sup>-alkylguanine-DNA-alkyltransferase fails to repair O<sup>6</sup>-methylthymine and methyl phosphotriesters in DNA as efficiently as does the alkyltransferase from *E. coli*. *Carcinogenesis*, *6*, 949-953

- Yarosh, D.B., Hurst-Calderone, S., Babich, M. & Day, R.S. (1986) Inactivation of *O*<sup>6</sup>-methylguanine-DNA methyltransferase and sensitization of human tumour cells to killing by chloroethylnitrosourea by *O*<sup>6</sup>-methylguanine as the free base. *Cancer Res.*, *46*, 1663-1668
- Yeichiel, E. & Barenholz, Y. (1985) Relationships between membrane lipid composition and biological properties of rat myocytes. *J. biol. Chem.*, *260*, 9123-9131
- Ying, T., Sarma, D.S.R. & Farber, E. (1981) Role of acute necrosis in the induction of early steps in liver carcinogenesis by diethylnitrosamine. *Cancer Res.*, *41*, 2096-2102
- Yoo, J.S.H. & Yang, C.S. (1985) Enzyme specificity in the metabolic activation of *N*-nitrosodimethylamine to a mutagen for Chinese hamster V79 cells. *Cancer Res.*, *45*, 5569-5574
- Yoshida, K., Kosama, K., Kitabatake, M., Okuda, M. & Imai, U. (1980) Metabolic fate of nitric oxide. *Int. Arch. occup. environ. Health*, *46*, 71-77
- Yoshida, K., Kosama, K., Kitabatake, U. & Imai, U. (1983) Biotransformation of nitric oxide, nitrite and nitrate. *Int. Arch. occup. environ. Health*, *52*, 103-115
- Yu, M.C., Ho, J.H.C., Ross, R.K. & Henderson, B.E. (1981) Nasopharyngeal carcinoma in Chinese — salted fish or inhaled smoke? *Prev. Med.*, *10*, 15-24
- Yu, M.C., Ho, J.H.C., Henderson, B.E. & Armstrong, R.W. (1985) Epidemiology of nasopharyngeal carcinoma in Malaysia and Hong Kong. *Natl Cancer Inst. Monogr.*, *69*, 203-207
- Yu, M.C., Ho, J.H.C., Lai, S.H. & Henderson, B.E. (1986) Cantonese-style salted fish as a cause of nasopharyngeal carcinoma: report of a case-control study in Hong Kong. *Cancer Res.*, *46*, 956-961
- Zacharias, H., Geilhaupt, M., Meier, K. & Welge, K.H. (1980) Laser photofragment spectroscopy of the NO<sub>2</sub> dissociation at 337 nm. A non-statistical decay process. *J. Chem. Phys.*, *74*, 218-225
- Zaldivar, R. (1977) Nitrate fertilizers as environmental pollutants: positive correlation between nitrates (NaNO<sub>3</sub> and KNO<sub>3</sub>) used per unit area and stomach cancer mortality rates. *Experientia*, *33*, 264-265
- Zarbl, H., Sukumar, S., Arthur, A.V., Martin-Zanka, D. & Barbacid, M. (1985) Direct mutagenesis of H-ras-1 oncogenes by *N*-nitroso-*N*-methylurea during initiation of mammary carcinogenesis in rats. *Nature*, *315*, 382-385
- Zeisel, S.H. & DaCosta, K.-A. (1986) Increase in human exposure to methylamine precursors of *N*-nitrosamines after eating fish. *Cancer Res.*, *46*, 6136-6138
- Zeisel, S.H., DaCosta, K.-A. & Fox, J.G. (1985) Endogenous formation of dimethylamine. *Biochem. J.*, *232*, 403-408
- Zeller, W.J., Lijinsky, W. & Eisenbrand, G. (1985) Antitumor activity of 1-nitroso-1-(2-chloroethyl)-3-(hydroxyalkyl)-ureas. *J. Cancer Res. clin. Oncol.*, *109*, 37
- Ziebarth, D. & Teichmann, B. (1980) Nitrosation of orally administered drugs under simulated stomach conditions. In: Walker, E.A., Griciūtė, L., Castegnaro, M. & Börzsönyi, M., eds, *N-Nitroso Compounds: Analysis, Formation and Occurrence (IARC Scientific Publications No. 31)*, Lyon, International Agency for Research on Cancer, pp. 231-244
- Ziegler, R.G., Morris, L.E. & Blot, W.J. (1981) Esophageal cancer among black men in Washington DC. II. Role of nutrition. *J. natl Cancer Inst.*, *67*, 1199-1206
- Ziegler, R.G., Mason, T.J., Stemhagen, A., Hoover, R., Schoenberg, J.B., Gridley, G., Virgo, P.W. & Fraumeni, J.F. (1986) Carotenoid intake, vegetables and the risk of lung cancer among white men in New Jersey. *Am. J. Epidemiol.*, *123*, 1080-1093
- Zurlo, J., Curphey, T.J., Hiley, R. & Longnecker, D.S. (1982) Identification of *N*-7-carboxymethylguanine in DNA from pancreatic acinar cells exposed to azserine. *Cancer Res.*, *42*, 1286-1288

**ORGANIZERS AND SPONSORS OF THE  
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## **LIST OF PARTICIPANTS**

## LIST OF PARTICIPANTS

- F. ADLKOEFER**  
Gazellenhaus 40, 2 Hamburg 54, FRG
- L. AIROLDI**  
Laboratory of Environmental Pharmacology & Toxicology, Mario Negri Institute for Pharmacological Research, Via Eritrea 62, 20157 Milan, Italy
- R.A. ANDERSEN**  
Agricultural Research Service, US Department of Agriculture, Department of Agronomy, University of Kentucky, Lexington, KY 40546-0091, USA
- S. ANTHONY**  
International Agency for Research on Cancer, 150 cours Albert Thomas, 69372 Lyon Cedex 08, France
- K. APPEL**  
Max von Pettenkofer Institute, Federal Health Office, Thieleallee, 1000 Berlin (West) 33, FRG
- M.C. ARCHER**  
The Beatson Institute for Cancer Research, Wolfson Laboratory for Molecular Pathology, Garscube Estate, Switchback Road, Bearsden, Glasgow G61 1BD, Scotland, UK
- B. BARTSCH**  
International Agency for Research on Cancer, 150 cours Albert Thomas, 69372 Lyon Cedex 08, France
- P.M.G. BAVIN**  
Smith Kline & French Research Limited, The Frythe, Welwyn, Herts AL6 9AR, UK
- B. BEIJE**  
Department of Genetic and Cellular Toxicology, Wallenberg Laboratory, Stockholm University, S-106 91 Stockholm, Sweden
- S. BELINSKY**  
Laboratory of Biochemical Risk Analysis, National Institute of Environmental Health Sciences, MD D4-04, PO Box 12233, Research Triangle Park, NC 27709, USA
- T. BELLANDER**  
Department of Occupational Medicine, Block F, University Hospital, S-221 85 Lund, Sweden
- M.R. BERGER**  
Institute of Toxicology and Chemotherapy, German Cancer Research Institute, Im Neuenheimer Feld 280, 6900 Heidelberg, FRG
- D. BETHGE**  
Institute of Organic Chemistry, University of Hamburg, Martin-Luther King Platz 6, D-2000 Hamburg 13, FRG
- V. BHIDE**  
Carcinogenesis Division, Cancer Research Institute, Tata Memorial Centre, Parel, Bombay 400012, India

## LIST OF PARTICIPANTS

- O. BÖCKMAN Norsk Hydro Research Centre, N-3901 Prosgrunn, Norway
- P. BOGOVSKI  
Institute of Experimental and Clinical Medicine, 41 Hiiu Street, Tallinn 200107, Estonia, USSR
- M. BONFANTI  
Laboratory of Environmental Pharmacology & Toxicology, Mario Negri Institute for Pharmacological Research, Via Eritrea 62, 20157 Milan, Italy
- M. BÖRZSÖNYI  
National Institute of Hygiene, Gyali ut 2-6, 1966 Budapest, Hungary
- L.W. VAN BROEKHOVEN  
Center for Agrobiological Research, PO Box 14, 6700 AA Wageningen, The Netherlands
- K.D. BRUNNEMANN  
Naylor Dana Institute for Disease Prevention, American Health Foundation, Dana Road, Valhalla NY 10595, USA
- G.T. BRYAN  
Department of Human Oncology, University of Wisconsin Clinical Cancer Center, K4/528 CSC, 600 Highland Avenue, Madison, WI 53792, USA
- S. CALMELS  
International Agency for Research on Cancer, 150 cours Albert Thomas, 69372 Lyon Cedex 08, France
- M. CASTEGNARO  
International Agency for Research on Cancer, 150 cours Albert Thomas, 69372 Lyon Cedex 08, France
- A. CASTONGUAY  
School of Pharmacy, Laval University, Quebec, PQ G1K 7P4, Canada
- C. CAYGILL  
PHLS Communicable Disease Surveillance Centre, 61 Colindale Avenue, London NW9 5HT, UK
- B. CHALLIS  
Chemistry Department, Imperial College, London SW7 2AZ, UK
- J. CHEN  
Institute of Nutrition & Food Hygiene, China Academy for Preventive Medicine, 29 Nan Wei Road, Beijing, China
- N.W. CHOI  
Department of Epidemiology & Biostatistics, Manitoba Cancer Treatment & Research Foundation, Winnipeg, Manitoba, Canada
- F.-L. CHUNG  
Naylor Dana Institute for Disease Prevention, American Health Foundation, Dana Road, Valhalla NY 10595, USA
- F.G. COLBY  
Frank Colby & Associates, Inc., 28th Floor, 9+ Park Avenue, New York, NY 10016, USA
- J. CONRAD  
Institute of Food Chemistry & Environmental Toxicology, University of Kaiserslautern, Erwin-Schrödinger-Strasse, 6750 Kaiserslautern, FRG

- P. CORREA**  
Department of Pathology, Louisiana State University Medical Center, 1901 Perdido Street, New Orleans, LA 70112, USA
- V.M. CRADDOCK**  
MRC Toxicology Unit, Medical Research Council Laboratories, Woodmansterne Road, Carshalton, Surrey SM5 4EF, UK
- A. VON DER DECKEN**  
The Wenner-Gren Institute, University of Stockholm, Biology F 3, S-106 91 Stockholm Sweden
- L. DEN ENGELSE**  
Division of Chemical Carcinogenesis, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands
- E. DENKEL**  
Institute of Food Chemistry & Environmental Toxicology, University of Kaiserslautern, Erwin-Schrödinger-Strasse, 6750 Kaiserslautern, FRG
- G. DESCOTES**  
Claude Bernard University, Campus la Doua, 43 Bld du 11 Novembre 1918, 69622 Villeurbanne Cedex, France
- H.E. DRIVER**  
MRC Toxicology Unit, Medical Research Council Laboratories, Woodmansterne Road, Carshalton, Surrey SM5 4EF, UK
- G. EISENBRAND**  
Institute of Food Chemistry & Environmental Toxicology, University of Kaiserslautern, PO Box 3049, Erwin-Schrodinger Strasse, 6750 Kaiserslautern, FRG
- G. ELLEN**  
National Institute of Public Health and Environmental Hygiene, PO Box 1, 3720 BA Bilthoven, The Netherlands
- P.B. FARMER**  
MRC Toxicology Unit, Medical Research Council Laboratories, Woodmansterne Road, Carshalton, Surrey SM5 4EF, UK
- J.G. FARRELLY**  
National Cancer Institute, Frederick Cancer Research Facility, LBI-Basic Research Program, PO Box B, Frederick MD 21701, USA
- Y. FELLION**  
Richardson Vicks Research Centre, 10 rue de Citeaux, Paris 75012, France
- W. FIDDLER**  
US Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, 600 E Mermaid Lane, Philadelphia, PA 19118, USA
- W. FINK**  
Philip Morris Europe, Research Department, PO Box 11, Quai Jeanrenaud 53, 2003 Neuchatel, Switzerland
- U. FISCHER**  
F. Hoffmann-La Roche & Co Ltd, Department PF/C, Bldg 15, 4002 Basel, Switzerland

**L.Y.Y. FONG**

Department of Biochemistry, Faculty of Medicine, University of Hong Kong, Li Shu Fan Building, Sassoon Road, Hong Kong

**D. FORMAN**

Imperial Cancer Research Fund, Cancer Epidemiology & Clinical Trials Unit, Gibson Laboratories, Radcliffe Infirmary, Oxford OX2 6HE, UK

**E. FREI**

Institute of Toxicology & Chemotherapy, German Cancer Research Center, Im Neuenheimer Feld 280, 6900 Heidelberg, FRG

**H.W. GAISCH**

Fabriques de Tabac Réunion SA, 2003 Neuchatel, Switzerland

**M.O. GARCÍA-ROCHÉ**

Institute of Nutrition and Food Hygiene, Infanta 1158, City of Havana, Cuba

**U. GOFF**

Thermedics Inc., 470 Wildwood Street, PO Box 2999, Woburn, MA 01888-1799, USA

**B. GOLD**

The Eppley Institute for Research on Cancer and Allied Diseases, University of Nebraska Medical Center, 42 and Dewey Avenue, Omaha, NE 68105, USA

**R. GRAFSTRÖM**

Department of Toxicology, Karolinska Institute, Box 60 400, S-104 01 Stockholm, Sweden

**Y. GRANJARD**

International Agency for Research on Cancer, 150 cours Albert Thomas, 69372 Lyon Cedex 08, France

**D.W. GRUENWEDEL**

College of Agricultural & Environmental Sciences, Department of Food Science & Technology, University of California, 1480 Chemistry Annex, Davis, CA 95616, USA

**J. GRY**

Institute of Toxicology, National Food Agency, Mørkhøj Bygade 19, 2860 Søborg, Denmark

**J.B. GUTTENPLAN**

Department of Biochemistry, New York University Dental Center, 345 E 24th Street, New York, NY 10010, USA

**C.N. HALL**

Norman Tanner Gastroenterology Unit, St James Hospital, Balham, London SW12 8HW, UK

**M. HAMANO**

Tokyo Kaseigakuin University, 22 Sanbancho, Chiyoda-ku, Tokyo 102, Japan

**C.C. HARRIS**

Laboratory of Human Carcinogenesis, Building 37, Room 2C07, National Cancer Institute, Bethesda, MD 20892, USA

**H.J. HAUSSMANN**

Institute of Toxicology, University of Tübingen, Wilhelmstrasse 56, D-7400 Tübingen 1, FRG

**D. HAVERY**

Food & Drug Administration, 200 'C' Street SW, HFF-459, Washington DC 20204, USA

**H. HAYATSU**

Faculty of Pharmaceutical Sciences, Okayama University, Tsushima, Okayama 700, Japan

- S.S. HECHT**  
Naylor Dana Institute for Disease Prevention, American Health Foundation, Dana Road,  
Valhalla NY 10595, USA
- V. HEEMANN**  
BAT Cigarettenfabriken GmbH, Bahrenfelder Chaussee 139, 2000 Hamburg 50, FRG
- E. HESELTINE**  
International Agency for Research on Cancer, 150 cours Albert Thomas, 69372 Lyon Cedex 08,  
France
- J.-M. HIRSCH**  
Oral Surgery, Emergency Dental Clinic, Stampgatan 2, 411 01 Göteborg, Sweden
- D. HOFFMANN**  
Naylor Dana Institute for Disease Prevention, American Health Foundation, Dana Road,  
Valhalla NY 10595, USA
- J.H. HOTCHKISS**  
Department of Food Science, Stocking Hall, Cornell University, Ithaca, NY 14853, USA
- C. JANZOWSKI**  
Institute of Food Chemistry & Environmental Toxicology, University of Kaiserslautern, Erwin-  
Schrodinger Strasse, 6750 Kaiserslautern, FRG
- L. JOHANSSON**  
Swedish Tobacco Co., Box 17007, 104 62 Stockholm, Sweden
- R. JOHNSON**  
Thermedics Inc., 470 Wildwood Street, PO Box 2999, Woburn, MA 01888-1799, USA
- T. KAWABATA**  
Department of Biomedical Research on Food, National Institute of Health, 2-10-35 Kamiosaki,  
Shinagawa-ku, Tokyo 141, Japan
- T. KAWANISHI**  
Division of Pharmacology, Biological Safety Research Center, National Institute of Hygienic  
Sciences, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158, Japan
- L.K. KEEFER**  
Chemistry Section, Laboratory of Comparative Carcinogenesis, National Cancer Institute,  
Frederick Cancer Research Facility, Bldg 538, Frederick, MD 21701-1013, USA
- M. KLAUDE**  
The Wenner-Gren Institute, University of Stockholm, Biology F3, S-106 91  
Stockholm, Sweden
- P. KLEIHUES**  
Laboratory of Neuropathology, Department of Pathology, University of Zurich,  
Schmelzbergstrasse 12, CH-8091 Zurich, Switzerland
- R.G. KLEIN**  
Institute for Toxicology & Chemotherapy, German Cancer Research Center, Im Neuenheimer  
Feld 280, 6900 Heidelberg, FRG
- H. KLUS**  
Austria Tabakwerke, Research and Development, Hasnerstrasse 124a, A-1160 Vienna, Austria

**T.M. KNIGHT**

Imperial Cancer Research Fund, Gibson Building, Radcliffe Infirmary, Oxford OX2 6HE, UK

**S.R. KOEPKE**

Chemical Carcinogenesis Program, LBI-Basic Research Program, National Cancer Institute, Frederick Cancer Research Facility, PO Box B, Frederick, MD 21701, USA

**Y. KONISHI**

Department of Oncological Pathology, Cancer Center, Nara Medical College, 840 Shijo-cho, Kashihara, Nara 634, Japan

**N. KOPPANG**

National Veterinary Institute, Postboks 8156 Dep, N-0033 Oslo 1, Norway

**P. KROGH**

Department of Microbiology, Royal Dental College, Copenhagen, Denmark

**A. KUNTZ**

Free University of Berlin, Clinic for Diseases of the Teeth, Mouth and Jaw — Oral Surgery, Föhlerstrasse 15, D-1000 Berlin (West) 65, FRG

**S.A. KYRTOPOULOS**

The National Hellenic Research Foundation, Institute of Biological Research 48 Vassileos Constantinou Avenue, Athens 116-35, Greece

**S. LEACH**

PHLS Centre for Applied Microbiology & Research, Bacterial Metabolism Research Laboratory, Porton Down, Salisbury, Wilts SP4 0JH, UK

**M. LEHMANN**

Experimental Toxicology, Schering AG, Muellerstrasse 170-178, D-1000 Berlin (West) 65, FRG

**R.N. LOEPPKY**

Department of Chemistry, 123 Chemistry Building, University of Missouri-Columbia, Columbia, MO 65211, USA

**P.D. LOTLIKAR**

Fels Research Institute, Temple University Medical School, Philadelphia, PA 19140, USA

**S.H. LU**

Department of Chemical Etiology & Carcinogenesis, Cancer Institute, Chinese Academy of Medical Sciences, Beijing, China

**W.K. LUTZ**

Institute of Toxicology, Swiss Institute of Technology & University of Zurich, Schorenstrasse 16, CH-8603 Schwerzenbach bei Zürich, Switzerland

**P. MAGEE**

Fels Research Institute, Temple University School of Medicine, Philadelphia, PA 19140, USA

**M.A. MARLETTA**

Department of Applied Biological Sciences, Rm 56-229, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 01239, USA

**R. MASSEY**

Ministry of Agriculture, Fisheries & Food, Food Science Laboratory, Haldin House, Old Bank of England Court, Queen Street, Norwich NR2 4SX, UK

**C.J. MICHEJDA**

Laboratory of Chemical and Physical Carcinogenesis, LBI-Basic Research Program, National Cancer Institute, Frederick Cancer Research Facility, PO Box B, Frederick MD 21701, USA

**S. MIRVISH**

The Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, 32nd and Dewey Avenue, Omaha, NE 68105, USA

**M. MIWA**

National Food Research Institute, 2-1-2 Yatabe, Kannondai, Tsukuba, Ibaraki 305, Japan

**M. MOCHIZUKI**

Kyoritsu College of Pharmacy, Shibakoen 1-5-30, Minato-ku, Tokyo 105, Japan

**M. MOELLER**

Norsk Hydro Research Centre, N-3901 Porsgrunn, Norway

**Y. MORI**

Laboratory of Radiochemistry, Gifu Pharmaceutical University, 6-1 Mitahorahigashi 5-chome, Gifu 502, Japan

**R. MUGGLI**

Department of Human Nutrition and Health, F. Hoffmann Laroche, 4002 Basel, Switzerland

**D. NAGEL**

The Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, 32nd and Dewey Avenue, Omaha, NE 68105, USA

**N. NAIR**

Carcinogenesis Division, Cancer Research Institute, Tata Memorial Centre, Parel, Bombay 400012, India

**M. NAKAMURA**

Department of Food & Drug Science, Kanagawa Prefectural Public Health Laboratory, 52-2 Nakao-cho, Asahi-ku, Yokohama 241, Japan

**C.M. O'DONNELL**

Department of Surgery, University of Liverpool, PO Box 147, Liverpool L69 3BX, UK

**H. OHSHIMA**

International Agency for Research on Cancer, 150 cours Albert Thomas, 69372 Lyon Cedex 08, France

**M. OKADA**

Tokyo Biochemical Research Institute, Takada 3-41-8, Toshima-ku, Tokyo 171, Japan

**I.K. O'NEILL**

International Agency for Research on Cancer, 150 cours Albert Thomas, 69372 Lyon Cedex 08, France

**B.G. ÖSTERDAHL**

The National Food Administration, Nutritional Laboratory, Box 622, 751 26 Uppsala, Sweden

**W. PARZEFALL**

Institute for Tumour Biology and Cancer Research, University of Vienna, Borschkegasse 8a, A-1090 Vienna, Austria

**R. PASTORELLI**

Laboratory for Environmental Pharmacology & Toxicology, Mario Negri Institute for Pharmacological Research, Via Eritrea 62, 20157 Milan, Italy

**A.E. PEGG**

Department of Physiology, The Milton S. Hershey Medical Center, The Pennsylvania State University, PO Box 850, Hershey, PA 17033, USA



**J. PFAB**

Department of Chemistry, Heriot-Watt University, Riccarton, Edinburgh EH14 4AS, Scotland, UK

**B. PIGNATELLI**

International Agency for Research on Cancer, 150 cours Albert Thomas, 69372 Lyon Cedex 08, France

**D. PISKORSKA**

Institute of Organic Chemistry, University of Gdansk, Tetmajera 4A-13, 80313 Gdansk, Poland

**S. POIRIER**

International Agency for Research on Cancer, 150 cours Albert Thomas, 69372 Lyon Cedex 08, France

**J.R.A. POLLOCK**

Pollock and Pool Ltd, Ladbrooke Close, Woodley, Reading RG5 4DX, Berks, UK

**B.L. POOL**

Institute for Toxicology & Chemotherapy, German Cancer Research Center, Im Neuenheimer Feld 280, 6900 Heidelberg 1, FRG

**V. PREAT**

Unit of Toxicology & Cancerological Biochemistry, Catholic University of Louvain, UCL 7369, B-1200 Brussels, Belgium

**S. PRESTON-MARTIN**

Department of Preventive Medicine, University of Southern California, School of Medicine, 1420 San Pablo Street, Los Angeles CA 90033-9987, USA

**R. PREUSSMANN**

Institute for Toxicology & Chemotherapy, German Cancer Research Center, Im Neuenheimer Feld 280, 6900 Heidelberg, FRG

**D.J. REED**

Environmental Health Sciences Center, Oregon State University, Weniger 317, Corvallis, OR 97331, USA

**P.I. REED**

Gastrointestinal Unit, Wexham Park Hospital, Slough SL2 4HL, Berks, UK

**H. REMMER**

Institute of Toxicology, University of Tübingen, Wilhelmstrasse 56, D-7400 Tübingen 1, FRG

**E. RICHTER**

Walther Straub Institute for Pharmacology & Toxicology, Ludwig-Maximilians University, Nussbaumstrasse 26, D-8000 Munich 2, FRG

**P.D. ROSS**

University of Hawaii at Manoa, Cancer Research Center of Hawaii, 1236 Lauhala Street, Honolulu, Hawaii 96813, USA

**E.B. SANSONE**

National Cancer Institute, Frederick Cancer Research Facility, PO Box B, Frederick, MD 21701, USA

**R.S. SAVALGI**

Medicine 2, Jenner Wing, St George's Hospital Medical School, Cranmer Terrace, London SW17 0RE, UK

- R. A. SCANLAN**  
Department of Food Science & Technology, Oregon State University, Corvallis, OR 97331, USA
- E. SCHERER**  
Division of Chemical Carcinogenesis, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands
- G. SCHERER**  
Forschungsgesellschaft Rauchen und Gesundheit mbH, Mittelweg 17, D-2000 Hamburg 13, FRG
- K. SCHLEMMER**  
Institute of Food Chemistry & Environmental Toxicology, University of Kaiserslautern, PO Box 3049, Erwin-Schrödinger-Strasse, 6750 Kaiserslautern, FRG
- D. SCHMÄHL**  
Institute for Toxicology & Chemotherapy, German Cancer Research Center, Im Neuenheimer Feld 280, 6900 Heidelberg, FRG
- J. SCHMEROLD**  
Laboratory of Neuropathology, Department of Pathology, University of Zurich, Schmelzbergstrasse 12, CH-8091 Zürich, Switzerland
- P. SCHMEZER**  
Institute for Toxicology & Chemotherapy, German Cancer Research Center, Im Neuenheimer Feld 280, 6900 Heidelberg 1, FRG
- J. SCHMID**  
Dr Karl Thomae GmbH, Postfach 1755, D-7950 Biberach/Riss 1, FRG
- H.M. SCHULLER**  
Experimental Oncology Laboratory, Department of Pathobiology, College of Veterinary Medicine, University of Tennessee, Knoxville, TN 37901, USA
- R. SCHULTE-HERMANN**  
Institute for Tumour Biology and Cancer Research, University of Vienna, Borschkegasse 8a, A-1090 Vienna, Austria
- S.E. SHEPHARD**  
Institute of Toxicology, Swiss Institute of Technology and University of Zurich, Schorenstrasse 16, CH-8603 Schwerzenbach, Switzerland
- H. SHIMADA**  
Faculty of Pharmaceutical Sciences, Okayama University, Tsushima, Okayama 700, Japan
- D. SHUKER**  
International Agency for Research on Cancer, 150 cours Albert Thomas, 69372 Lyon Cedex 08, France
- B. SINGER**  
Donner Laboratory, University of California, Berkeley CA 94720, USA
- B. SPIEGELHALDER**  
Institute for Toxicology & Chemotherapy, German Cancer Research Center, Im Neuenheimer Feld 280, 6900 Heidelberg, FRG
- S. SRIANUJATA**  
Research Center, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Rama 6 Road, Bangkok 10400, Thailand
- W. STAHL**  
Institute of Food Chemistry & Environmental Toxicology, University of Kaiserslautern, Box 3049, Erwin-Schrödinger-Strasse, 6750 Kaiserslautern, FRG

**B.W. STEWART**

Children's Leukaemia & Cancer Research Unit, Prince of Wales Children's Hospital, Randwick,  
NSW 2031, Australia

**T. SUGIMURA**

National Cancer Center Research Institute, 1-1 Tsukiji 5-chome, Chuo-ku, Tokyo 104, Japan

**E. SUZUKI**

Tokyo Biochemical Research Institute, Takada 3-41-8 Toshima-ku, Tokyo 171, Japan

**P. SWANN**

Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, Mortimer Street,  
London W1P 7PN, UK

**J.A. SWENBERG**

Chemical Industry Institute of Toxicology, PO Box 12137, Research Triangle Park, NC 27709,  
USA

**W. TANG**

Institute of Food Chemistry & Environmental Toxicology, University of Kaiserslautern, Box  
3049, Erwin-Schrodinger-Strasse, 6750 Kaiserslautern, FRG

**S.R. TANNENBAUM**

Department of Nutrition & Food Science, Rm 56-311, Massachusetts Institute of Technology, 77  
Massachusetts Avenue, Cambridge MA 02139, USA

**H. TJALVE**

Department of Pharmacology & Toxicology, Swedish University of Agricultural Sciences,  
Uppsala Biomedical Centre, Box 573, S-751 23 Uppsala, Sweden

**L. TOMATIS**

International Agency for Research on Cancer, 150 cours Albert Thomas, 69372 Lyon Cedex 08,  
France

**A.R. TRICKER**

Institute for Toxicology & Chemotherapy, German Cancer Research Center, Im Neuenheimer  
Feld 280, D-6900 Heidelberg, FRG

**M. TSUDA**

National Cancer Center Research Institute, 1-1 Tsukiji 5-chome, Chuo-ku, Tokyo 104, Japan

**C.-D. WACKER**

Institute for Toxicology & Chemotherapy, German Cancer Research Center, Im Neuenheimer  
Feld 280, D-6900 Heidelberg, FRG

**K. WAKABAYASHI**

Carcinogenesis Division, National Cancer Center Research Institute, 1-1, Tsukiji 5-chome,  
Chuo-ku, Tokyo 104, Japan

**C.L. WALTERS**

Department of Biochemistry, University of Surrey, Guildford, Surrey, UK

**J. WARE**

Department of Surgery, Karolinska Institute, S104 01 Stockholm, Sweden

- S. WATSON  
Strathisla-Glenlivet Distillery, Seafield Avenue, Keith, Banffshire, UK
- J. WERRINGLOER  
Department of Toxicology, University of Tübingen, Wilhelmstrasse 56, D-7400 Tübingen, FRG
- M. WIESSLER  
Institute for Toxicology & Chemotherapy, German Cancer Research Center, Im Neuenheimer  
Feld 280, 6900 Heidelberg, FRG
- C. WILD  
International Agency for Research on Cancer, 150 cours Albert Thomas, 69372 Lyon Cedex 08,  
France
- J.S. WISHNOK  
Department of Applied Biological Sciences, Rm 56-313, Massachusetts Institute of Technology,  
77 Massachusetts Avenue, Cambridge, MA 02139, USA
- H. WRBA  
Institute for Tumour Biology and Cancer Research, University of Vienna, Borschkegasse 8a,  
A-1090 Vienna, Austria
- C.S. YANG  
Department of Biochemistry, The University of Medicine & Dentistry of New Jersey, New Jersey  
Medical School, Newark, NJ 07103, USA
- K. YANO  
Department of Chemistry, Saitama Medical School, 981 Kawakado, Moroyama-cho, Iruma-  
gun, Saitama 350-04, Japan
- M.C. YU  
Department of Preventive Medicine, University of Southern California, School of Medicine,  
NOR 722, 2025 Zonal Avenue, Los Angeles, CA 90033, USA

## NOMENCLATURE AND ABBREVIATIONS

Following the system of nomenclature for *N*-nitroso compounds proposed in the Proceedings of the Fifth Meeting in this series, which was based on the IUPAC system of nomenclature, additional proposals are made for systematization of nomenclature and abbreviations of these compounds.

### *N*-NITROSAMINES

1. As in existing recommendations, the *N*-NO radical is always stated first (abbreviations commence with 'N'); the parts joined to the amine nitrogen follow; and, where appropriate, the names terminate with 'amine' and the abbreviation with 'A'.

2. The parts joined to the amine nitrogen are placed in the following order, both in nomenclature and in abbreviation:

- (i) aliphatic and alicyclic radicals
- (ii) aromatic radicals
- (iii) non-aromatic heterocyclic radicals
- (iv) oxidized radicals
- (v) alkene radicals
- (vi) other types or derivatives.

When there are two radicals of the same type, the larger one is given first (by number of carbon atom, then mass).

3. Unless otherwise specified, alkane radicals are normal and unbranched. Branched alkane radicals are denoted by placing *i*(iso), *s*(sec) and *t*(tert) before the radical name and before the radical abbreviation. The position of substituents on these chains is specified, giving the carbon position before the derivative. Note that an  $\alpha$ -keto function turns the amine into an amide, for which a variation in nomenclature and abbreviation is proposed to reflect the significant alteration in chemical properties (see below).

4. The following standard abbreviations are reserved:

D = di or bis (i.e., two radicals of same type attached to the amine nitrogen, as in NDMA and NDHPA).

M, E, P, B, Ph, Bz are reserved for methyl, ethyl, propyl, butyl, phenyl, benzyl, respectively.

PIP, PYR, MOR, PZ, SAR, PRO, THZ and AZ are reserved for piperidine, pyrrolidine, morpholine, piperazine, sarcosine, proline, thiazolidine and azetidine, respectively.

5. Derivation of radicals by hydroxy, keto or acetoxy groups is covered by placing H, O or Ac in front of the respective radical abbreviation.

6. The abbreviation NDELA is retained, due to its widespread usage.

**N-NITROSAMIDES**

1. Instead of using ammonia as the root for nomenclature, an amide is taken, so that, for example, an  $\alpha$ -keto propylamine part of a molecule is a propionamide radical. This complete radical is then placed at the end of the nomenclature and abbreviation, e.g., '-propionamide' and 'PAd'. The initial '*N*-nitroso' and 'N' are retained as with *N*-nitrosamines.

2. As only one more radical can be attached to the nitrogen, it is suggested that this be inserted between the '*N*-nitroso' and '-amide' parts, irrespective of its nature. In the case of nitrosoureas, however, the nomenclature ends with *-N*-nitrosourea and the abbreviations with -NU. For nitrosourethanes, use -NUT.

3. In all other respects the same terms are used as for *N*-nitrosamines.

**N-NITRAMINES**

It is proposed to use the same systematic nomenclature as for *N*-nitrosamines but to represent *N*-nitro as NT at the beginning, e.g., *N*-nitrodimethylamine = NTDMA.

**EXAMPLES**

<i>N</i> -nitrosodimethylamine	NDMA
<i>N</i> -nitrosodi- <i>n</i> -butylamine	NDBA
<i>N</i> -nitrosodi-isobutylamine	NDi-BA
<i>N</i> -nitrosoethylmethylamine	NEMA
<i>N</i> -nitrosopyrrolidine	NPYR
<i>N</i> -nitrosomorpholine	NMOR
<i>N</i> -nitrosohydroxyproline	NHPRO
<i>N</i> '-nitrosornicotine	NNN
<i>N</i> -nitrosodiethanolamine	NDELA
<i>N</i> -nitrosopropyl(2-hydroxypropyl)amine	NPHPA
<i>N</i> -nitrosomethyl(2-oxobutyl)amine	NMOBA
<i>N</i> -nitrosomethylbutyramide[= <i>N</i> -nitrosomethyl(1-oxobutyl)amine]	NMBAd
<i>N</i> -nitrosoethylvinylamine	NEVA
<i>N</i> -nitroso(2-hydroxypropyl)(2-oxopropyl)amine	NEHPOPA
<i>N</i> -nitrosobis(2-hydroxypropyl)amine	NDHPA
<i>N</i> -butyl- <i>N</i> -nitrosourea	BNU

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