

3. Studies of Cancer in Experimental Animals

In this section, only relevant studies on oestrogens and progestogens alone and in combination that were published subsequent to or not included in Volume 21 of the IARC Monographs (IARC, 1979) are reviewed in detail. Studies reviewed previously are summarized briefly.

3.1 Oestrogen–progestogen combinations

3.1.1 *Studies reviewed previously*

Mouse

The results of studies reviewed previously (Committee on Safety of Medicines, 1972; IARC, 1979) on the carcinogenicity of combinations of oestrogens and progestogens in mice are as follows:

Chlormadinone acetate in combination with mestranol tested by oral administration to mice caused an increased incidence of pituitary adenomas in animals of each sex. Oral administration of chlormadinone acetate in combination with ethinyloestradiol to mice resulted in an increased incidence of mammary tumours in intact and castrated males.

After oral administration of ethynodiol diacetate and mestranol to mice, increased incidences of pituitary adenomas were observed in animals of each sex. The combination of ethynodiol diacetate plus ethinyloestradiol, tested by oral administration to mice, increased the incidences of pituitary adenomas in animals of each sex and of malignant tumours of connective tissues of the uterus.

Lynoestrenol in combination with mestranol was tested in mice by oral administration. A slight, nonsignificant increase in the incidence of malignant mammary tumours was observed in females which was greater than that caused by lynoestrenol or mestranol alone.

The combination of megestrol acetate plus ethinyloestradiol, tested by oral administration to mice, caused an increased incidence of malignant mammary tumours in animals of each sex.

Norethisterone acetate plus ethinyloestradiol, tested by oral administration to mice, increased the incidences of pituitary adenomas in animals of each sex, but the incidences were comparable to those induced by ethinyloestradiol alone. The combination of norethisterone plus ethinyloestradiol was also tested in mice by oral administration; an increased incidence of pituitary adenomas was observed in females. Norethisterone plus mestranol increased the incidences of pituitary adenomas in animals of each sex.

Norethynodrel in combination with mestranol was tested by oral administration in mice. Increased incidences of vaginal or cervical tumours were found in female mice and of pituitary adenomas in males and females. In female mice, an increased incidence of

malignant mammary tumours was observed, but the incidence was not greater than that seen with norethynodrel alone. In castrated male mice, the combined treatment resulted in an increased incidence of mammary tumours.

The combination of norgestrel plus ethinyloestradiol was tested in mice by oral administration; no increase in the incidence of tumours was observed.

Rat

The results of studies reviewed previously (Committee on Safety of Medicines, 1972; IARC, 1979) on the carcinogenicity in rats of several combinations are as follows:

Ethinodiol diacetate plus ethinyloestradiol was tested for carcinogenicity by oral administration to rats. The incidence of malignant mammary tumours was increased in animals of each sex. In combination with mestranol, the incidence of mammary tumours was increased in one study but not in another.

Lynoestrenol in combination with mestranol was tested in female rats by oral administration. No increase in tumour incidence was observed.

Megestrol acetate plus ethinyloestradiol was tested by oral administration to rats. The incidence of benign liver tumours was increased in animals of each sex, but not to a level greater than that observed with ethinyloestradiol alone. In male rats, there was a small increase in the incidence of benign and malignant mammary tumours; females showed a small increase in the incidence of malignant mammary tumours.

The combination of noresthisterone acetate plus ethinyloestradiol, tested by oral administration to rats, increased the incidences of benign mammary tumours and liver adenomas in males. Norethisterone plus mestranol increased the incidence of malignant mammary tumours in female rats and increased the incidence of liver adenomas in males.

Norethynodrel in combination with mestranol was tested by oral administration to rats. In males, increased incidences of liver adenomas, pituitary adenomas and benign and malignant mammary tumours were observed, but the incidences were no greater than those with norethynodrel alone. In females, the incidences of pituitary adenomas and malignant mammary tumours were increased.

Norgestrel plus ethinyloestradiol, tested for carcinogenicity in rats by oral administration, caused a small increase in the incidence of benign mammary tumours in males.

Dimethisterone and oestradiol were tested in dogs, with no increase in the incidence of mammary tumours

3.1.2 *New studies*

(a) *Oral administration*

Rat

Schuppler and Gunzel (1979) summarized data from a study by the Committee on Safety of Medicines (1972) in the United Kingdom on the incidence of hepatocellular adenomas in groups of 24–124 male and female rats [strain not specified] treated orally with combinations of various oestrogens and progestogens at doses up to 400 times the

human contraceptive dose. The statistically significant increases indicated in their report are indicated by a '+' in Table 31.

Table 31. Effects of progestogen–oestrogen combinations on the incidence of hepatocellular adenomas in rats

Progestogen	Oestrogen	Ratio	Males	Females
Norethynodrel	Mestranol	66:1	+	–
		25:1	–	–
Norethisterone	Mestranol	20:1	+	–
Lynestrenol	Mestranol	33:1	Not tested	–
Megestrol acetate	Ethinylloestradiol	5:1	+	–
		80:1	+	–

From Schuppler & Gunzel (1979)

Groups of 10 female Sprague-Dawley rats, seven weeks of age, were treated with Enovid E (100 µg mestranol + 25 mg norethynodrel) in the diet for nine months, with daily intakes of 0.02–0.03 and 0.5–0.75 mg/kg bw. The numbers of altered γ -glutamyl transpeptidase (γ -GT)-positive hepatic foci, considered to be preneoplastic lesions, were counted at autopsy. A statistically significant ($p < 0.001$) increase in the number of foci (2.8 foci/cm²) was observed in comparison with untreated controls (0.2 foci/cm²). No increase in the incidence of hepatic nodules or carcinomas was observed at this time (Yager & Yager, 1980).

Female Wistar rats, 15–17 weeks of age, were treated with quingestanol acetate plus quinestrol, which are 3-cyclopentyl ether derivatives of norethisterone acetate and ethinylloestradiol, respectively, as a 2:1 mixture suspended in sesame oil containing piperidine (0.05% v/w) by stomach tube. A group of 75 rats was treated once weekly with 30 mg/kg bw, 60 rats were treated with 1.2 mg/kg bw per day, and 75 rats were used as vehicle controls; all treatments were given for 50 weeks, followed by 30 weeks of observation for reversibility of any lesions. Groups of 10 animals were killed at 25 and 50 weeks; at 66 weeks, five rats from each treatment group and three vehicle controls were killed, and at 80 weeks all survivors in the treated groups and 10 vehicle controls were killed. Treatment was associated with irreversible hair loss, a reversible decrease in body weight and reversible ataxia. The only treatment-related tumours were mammary masses and adenocarcinomas: at 40–50 weeks, the incidences of adenocarcinomas were 10/33 in rats at 30 mg/kg bw, 15/27 in those at 1.2 mg/kg bw and 1/12 in vehicle controls [statistics not specified]. After treatment was suspended, the incidences of mammary adenocarcinomas were reduced, with 1/8 at weeks 51–66 and 0/12 at weeks 67–80 in animals at 30 mg/kg bw, 3/7 and 2/8 at those times in animals at 1.2 mg/kg bw and 0/10 and 1/13 in vehicle controls (Lumb *et al.*, 1985).

Male and female Wistar rats, four weeks old, were given ethinylloestradiol (0.075 mg) + norethisterone acetate (6 mg) dissolved in olive oil by gavage daily, ethinylloestradiol +

norethisterone acetate + 10% ethanol in the drinking-water on five days a week, olive oil + ethanol or olive oil alone. The animals were treated for up to 12 months, with interim kills at two, four, six, eight and 12 months, when the livers were analysed for the presence of hepatocellular carcinomas and hyperplastic nodules. In females, ethinyloestradiol + norethisterone acetate induced a 100% incidence of hyperplastic nodules by four months and hepatocellular carcinomas in 2/25 animals at 12 months. Ethanol increased the incidence of hepatocellular carcinoma at 12 months to 9/22; no hyperplastic nodules or hepatocellular carcinomas were seen in the controls receiving ethanol alone. In males, ethinyloestradiol + norethisterone acetate induced hyperplastic nodules in 6/20 animals at 12 months but no hepatocellular carcinomas. Ethanol increased the incidence of hyperplastic nodules to 100%, beginning at four months, and that of hepatocellular carcinomas to 2/17 at 12 months. Again, ethanol alone had no effect on the incidences of hyperplastic nodules or hepatocellular carcinoma, but it enhanced nuclear and cytosolic oestrogen receptors and DNA adduct formation, as detected by ³²P-postlabelling (Yamagiwa *et al.*, 1991, 1994).

Monkey

Norlestrin (50:1 norethisterone acetate + ethinyloestradiol) was given to groups of 15–17 young adult female rhesus (*Macaca mulatta*) monkeys weighing 2.8–5.7 kg at the beginning of the study. Norlestrin powder was blended with soft fruit and vegetables and was administered over 10 years as 21 consecutive daily doses followed by seven days without treatment. The daily doses were 0, 0.051, 0.51 and 2.55 mg/kg bw which represented 0, 1, 10 and 50 times the human contraceptive dose. There were no effects on survival and no treatment-related alterations in coagulation or other clinical parameters. Only a few tumours appeared but were found in all groups (Fitzgerald *et al.*, 1982).

(b) Administration with known carcinogens

Mouse

Groups of 20 female B6AF₁ mice, 12 weeks of age, were treated with 3-methylcholanthrene by insertion of an impregnated silk thread into the cervical canal and through the uterine wall; a control group was treated with silk thread not impregnated with 3-methylcholanthrene. Pellets containing steroids were then implanted subcutaneously and renewed every three weeks for 15 weeks. The doses given every three weeks were 15 mg norethynodrel and 0.5 mg mestranol per mouse, alone and in combination. No tumours developed in mice that had not received 3-methylcholanthrene, but the steroids caused some histopathological changes in the mucosa of the cervix, uterus and vagina. The incidences of uterine adenoacanthomas were increased ($p < 0.01$) by all three treatments: control, 5/35; mestranol, 10/19; norethynodrel, 11/14; mestranol + norethynodrel, 10/18. Squamous-cell carcinomas of the cervix were observed in all groups, but the incidences were not statistically significantly increased in those receiving steroids (Blanzat-Reboud & Russfield, 1969).

The effects of two formulations, Ovral, consisting of 0.05 mg ethinyloestradiol + 0.5 mg norgestrel, and Noracycline, consisting of 0.05 mg ethinyloestradiol + 1 mg lynoestrenol, on

carcinomas of the uterine cervix induced by 3-methylcholanthrene were studied in groups of 10–30 Swiss albino female mice, eight to nine weeks of age. The mice treated with 3-methylcholanthrene received a sterile cotton thread impregnated with beeswax containing approximately 300 µg of the carcinogen into the uterine cervix. The oral contraceptive combinations were administered orally at doses of 1/2000th of a pill (0.025 µg ethinyloestradiol + 0.25 µg norgestrel), 1/200th of a pill (0.25 µg ethinyloestradiol + 2.5 µg norgestrel) and 1/20th of a pill (2.5 µg ethinyloestradiol + 25 µg norgestrel) of Ovral, and 1/2000th of a pill (0.025 µg ethinyloestradiol + 0.5 µg lynoestrol), 1/200th of a pill (0.25 µg ethinyloestradiol + 5 µg lynoestrol) and 1/20th of a pill (2.5 µg ethinyloestradiol + 50 µg lynoestrol) of Noracycline. Treatment was for 30, 60 or 90 days. Animals that did not receive 3-methylcholanthrene did not develop cervical tumours at any dose of oral contraceptive. In contrast, treatment with either formulation caused dose-dependent, biphasic effects on the incidence of squamous-cell carcinomas induced by 3-methylcholanthrene. At the two lower doses, they were protective in comparison with treatment with the carcinogen alone ($p < 0.05$), while at the high dose they enhanced carcinogenesis: The incidence of squamous-cell carcinomas was 6/23 with 3-methylcholanthrene alone and 8/17 with the high dose of Ovral at 90 days, although the difference was not indicated as being statistically significant. With Noracycline, the enhancement was statistically significant after both 60 days (13/24 versus 2/23, $p < 0.05$) and 90 days (12/19 versus 6/23, $p < 0.05$). At all doses and at all times, both formulations also significantly enhanced the incidence of cervical hyperplasia (Hussain & Rao, 1992).

In a more recent study with the same model, Ovral was administered to Swiss mice at the same two lower doses as the previous study, with 3-methylcholanthrene at a higher dose of 600 µg. The cotton thread was inserted into the right uterine horn. After 90 days, the incidence of tumours in the uterine endometrium was 8/15 in mice receiving the carcinogen alone and 1/16 in the group receiving the carcinogen + the 1/2000th dose of Ovral ($p < 0.05$). No tumours were seen in the group treated with 3-methylcholanthrene + Ovral at the 1/200th dose (Chhabra *et al.*, 1995).

Rat

Groups of 9–10 female Sprague-Dawley rats, seven weeks of age, were initiated by treatment with 5 mg/kg bw *N*-nitrosodiethylamine (NDEA) 24 h after partial hepatectomy. Twenty-four hours later they were fed a diet containing mestranol + norethynodrel at concentrations providing 0.02–0.03 and 0.5–0.75 mg/kg bw per day, respectively. After nine months, a statistically significant ($p < 0.001$) increase in the number of γ -GT-positive altered hepatic foci was observed (7.3 versus 0.3 foci/cm²), with no significant increase in the incidence of nodules or carcinomas (Yager & Yager, 1980).

Groups of female weanling Wistar rats received an intraperitoneal injection of 0 or 200 mg/kg bw NDEA. One month later, half the animals in each group (5–6 rats) received 1/10th of a tablet of Ovulen-50 (5 µg ethinyloestradiol + 100 µg ethynodiol diacetate) daily orally in 0.1 mL propylene glycol for 60 weeks; the other half of the rats

in each group received the vehicle. The livers were examined histochemically for γ -GT-positive foci and histologically. None of the rats developed liver tumours. In rats that had not been initiated with NDEA, Ovulen-50 increased the incidences of γ -GT-positive foci and of microscopic hyperplastic nodules in all five rats; such foci and nodules were not seen in other groups. The authors speculated that the absence of foci and nodules in NDEA-initiated rats with and without treatment with Ovulen-50 may have been due to an interplay of the drug-metabolizing enzymes and that the Ovulen-50 steroids were more rapidly metabolized by the NDEA-initiated rats (Annapurna *et al.*, 1988). [The Working Group noted that opposite effects, i.e. enhancement of foci and nodules in initiated livers by oral contraceptive steroids, have been seen in many other studies and that the results of this study must be considered an exception to the general finding and that they lack a mechanistic explanation.]

The results of the previous and new studies on oestrogen–progestogen combinations are summarized in Tables 32 and 33.

3.2 Oestrogens used in combined oral contraceptives

3.2.1 Studies reviewed previously

Mouse

Ethinylloestradiol administered to mice increased the incidence of pituitary adenomas and malignant mammary tumours in animals of each sex and the incidences of uterine and cervical tumours in females.

Mestranol increased the incidences of pituitary adenomas and malignant mammary tumours in animals of each sex.

Rat

When ethinylloestradiol was tested for carcinogenicity in rats, the incidences of liver adenomas were increased in animals of each sex and that of liver carcinomas in females.

Administration of mestranol increased the incidence of malignant mammary tumours in females in one of two treated groups.

3.2.2 New studies

(a) Oral administration

Mouse

Schuppler and Gunzel (1979) summarized data on the incidence of hepatic adenomas in groups of 40–120 male and female mice of three strains after oral administration of ethinylloestradiol or mestranol for 20 months at up to 400 times the human contraceptive dose. Only mice of strain BDH-SPF showed a small increase in incidence after receiving ethinylloestradiol.

Rat

Schuppler and Gunzel (1979) reported that the study of the Committee on Safety of Medicines (1972) found no increase in the incidence of liver adenoma in female rats

Table 32. Effects of combinations of various progestogens and oestrogens on tumour incidence in mice

Combination	Pituitary adenomas		Mammary tumours		Uterine tumours	Cervical/vaginal tumours	
	Male	Female	Benign (males)	Malignant			
				Male			Female
Chlormadinone acetate + mestranol	+	+					
Chlormadinone acetate + ethinyloestradiol			+/c				
Ethinodiol diacetate + mestranol	+	+					
Ethinodiol diacetate + ethinyloestradiol	+	+			+		
Lynoestrinol + mestranol					+/-		
Lynoestrinol + ethinyloestradiol + 3-methylcholanthrene						+ ^a	
Megestrol acetate + ethinyloestradiol				+	+		
Norethisterone acetate + ethinyloestradiol	+/?	+/?					
Norethisterone + ethinyloestradiol		+					
Norethisterone + mestranol	+	+					
Norethynodrel + mestranol	+	+	c		+/?	+	
Norethynodrel + mestranol + 3-methylcholanthrene					+	-	
Norgestrel + ethinyloestradiol + 3-methylcholanthrene						+ ^a	

+, increased tumour incidence; +/-, slightly increased tumour incidence; +/c, increased tumour incidence in intact and castrated animals; c, increased tumour incidence in castrated animals; +/?, increased tumour incidence, but not greater than that with the oestrogen or progestogen alone

^a Protection at doses 1/2000th and 1/200th that of a pill for women; enhancement at a dose of 1/20th that of a pill for women

Table 33. Effects of combinations of various progestogens and oestrogens on tumour incidence in rats

Combination	Pituitary adenomas		Mammary tumours			Liver				
	Male	Female	Benign (males)	Malignant		Adenoma		Carcinoma		Foci (females)
				Male	Female	Male	Female	Male	Female	
Ethinodiol diacetate + ethinylloestradiol				+	+					
Ethinodiol diacetate + mestranol				?	?					
Megestrol acetate + ethinylloestradiol			+/-	+/-	+/-	+/?	+/?			
Norethisterone acetate + ethinylloestradiol			+			+		-	+	
Norethisterone + mestranol					+	+	-			
Norethynodrel + mestranol	+/?	+	+/?	+/?	+	+/?	-	-	-	+
Norethynodrel + mestranol + <i>N</i> -nitrosodiethylamine							-		-	+
Norgestrel + ethinylloestradiol			+/-							

+, increased tumour incidence; +/-, slightly increased tumour incidence; +/?, increased tumour incidence, but not greater than that with the oestrogen or progestogen alone; ? conflicting; -, no effect

treated orally with mestranol. Of four studies on the incidences of hepatocellular adenoma and carcinoma in groups of 40–120 male and female rats treated orally with ethinyl-oestradiol at doses up to 400 times the human contraceptive dose, only one showed a statistically significant increase in the incidence of hepatocellular adenoma in males (0% controls, 15.3% treated) and in females (8% controls, 23.5% treated); the incidence of hepatocellular carcinoma was significantly increased only in females (0% controls, 7.4% treated).

Groups of female Sprague-Dawley rats received ethinyl-oestradiol or mestranol in the diet from about seven weeks of age at concentrations of 0.1 or 0.5 mg/kg diet (ppm) mestranol for nine and 12 months or 0.5 ppm ethinyl-oestradiol for nine months. The ingested doses were approximately equivalent to 6 or 30 $\mu\text{g}/\text{kg}$ bw per day or 3–15 times the human contraceptive dose. Ethinyl-oestradiol caused a statistically significant ($p < 0.05$) increase in the number of γ -GT-positive, altered hepatic foci, but not in the volume percentage of liver occupied by foci, after nine months. No increase in the incidence of nodules or carcinomas was observed. The high dose of mestranol had similar effects after nine months, but after 12 months, mestranol caused a statistically significant ($p < 0.05$) increase in both the number of altered hepatic foci, and the volume percentage of the liver occupied by foci showed a significant ($p < 0.05$) dose-response relationship. Furthermore, after 12 months, the high dose of mestranol caused a significant ($p < 0.05$) increase in the incidence of hepatic nodules and carcinomas combined (4/16 compared with 0/15 in controls) (Yager *et al.*, 1984).

Female Wistar rats, four weeks of age, were treated with 0 (control), 75 or 750 μg ethinyl-oestradiol in 0.5 mL olive oil by gavage daily for various times up to 12 months. By four months, the incidence of glutathione *S*-transferase-positive, altered hepatic foci, considered to be preneoplastic lesions, was 100% in both groups. At 12 months, hepatocellular carcinomas were found in 2/23 rats at 75 μg ethinyl-oestradiol and 10/26 at 750 μg , with none in 24 controls. This response correlated with increased oxidative damage to liver nuclear DNA. Antioxidant vitamins (vitamins C and E and β -carotene) slightly reduced the oxidative DNA damage, significantly reduced the number of altered hepatic foci and reduced the hepatocellular carcinoma incidence (Ogawa *et al.*, 1995).

Dog

Groups of 15 female beagles, 10–14 months of age at the start of the experiment, received mestranol at a dose of 0.02 or 0.05 mg/kg bw per day for cycles of 21 days followed by seven days with no drug; a group of 18 bitches served as controls. All of the animals were hysterectomized at two years of age. No mammary tumours were detected after five years (Kwapien *et al.*, 1980).

(b) *Subcutaneous implantation*

Rat

Holtzman (1988) studied the effects of retinyl acetate on ethinyl-oestradiol-induced mammary carcinogenesis. A group of 24 female ACI rats aged 59–65 days received sub-

cutaneously implanted ethinyloestradiol in cholesterol pellets (1 mg/20 mg pellet). All treated rats developed pituitary tumours, and 21/24 developed mammary gland carcinomas within 25 weeks. Retinyl acetate did not significantly decrease the mammary tumour incidence but reduced the tumour multiplicity by about 50%.

Hamster

A group of 15 male Syrian golden hamsters weighing 90–100 g received 20-mg pellets of ethinyloestradiol in the shoulder region. The pellets were replaced at three-month intervals and oestrogen treatment was continued for seven to eight months. Three animals developed microscopic renal-cell carcinomas. It was also reported that all 10 castrated male hamsters receiving similar treatment but fed 0.2% α -naphthoflavone developed hepatocellular carcinomas compared with 0/10 hamsters receiving only α -naphthoflavone in the diet (Li & Li, 1984).

(c) *Administration with known carcinogens*

Liver models

Mouse: Lee *et al.* (1989) compared the effects of several promoters, including ethinyloestradiol, in three strains of NDEA-initiated male mice. Six-week-old C3H/HeN (C3H), C57BL/6N (C57) and BALB/cA (BALB) mice underwent a two-thirds hepatectomy, followed 20 h later by an intraperitoneal injection of 20 mg/kg bw NDEA; 6 h later, the animals were given a diet containing phenobarbital at a concentration of 50 mg/kg diet (ppm), clofibrate at 1000 ppm and ethinyloestradiol at 10 ppm. The animals were killed after 20 weeks for detection of glucose 6-phosphatase-deficient, altered hepatic foci. The mouse strains differed widely with regard to the mean liver volume occupied by foci after receiving NDEA and in their sensitivity to promotion. The most sensitive strain was C3H. The mean volume ($\times 10^6 \mu\text{m}^3$) of the liver foci was 13.2 ± 1.8 in 18 mice that received NDEA only, 460 ± 72 in 20 mice given phenobarbital and 28 ± 6 in 20 mice given clofibrate; 11 mice given ethinyloestradiol showed no effect, the mean liver volume being 12 ± 10 . When the data were expressed as total volume of foci/cm³ liver $\times 10^6 \mu\text{m}^3$, ethinyloestradiol was seen to be protective, reducing the value of 710 ± 128 in 18 controls to 34 ± 22 . Similar results were found in C57 and BALB mice.

Female B6C3F₁ mice, 12 days of age, were treated with 5 mg/kg bw NDEA by intraperitoneal injection. At five to seven weeks of age, the mice were randomly assigned to groups of 12 which were exposed by inhalation to unleaded gasoline at 0, 292 or 2056 ppm for 6 h per day on five days per week for 16 weeks, to ethinyloestradiol in the diet at a concentration of 1 ppm or to 1 ppm ethinyloestradiol + 2056 ppm unleaded gasoline. Altered hepatic foci were determined in standard histological sections. The percentage of the liver volume occupied by foci was significantly reduced in ethinyloestradiol-treated mice, from 1.1 ± 0.7 in NDEA controls to 0.26 ± 0.31 ; however, the volume of foci was significantly increased by the high dose of unleaded gasoline, to 4.31 ± 2.51 , and further increased to 18 ± 5 by ethinyloestradiol + the high dose of unleaded gasoline (Standeven *et al.*, 1994).

Rat: Ethinyloestradiol and mestranol promoted the appearance of altered hepatic foci and the development of hepatic nodules (adenomas) and carcinomas in initiated male and female rats (Wanless & Medline, 1982; Mayol *et al.*, 1991; Hallstrom *et al.*, 1996; Yager & Liehr, 1996). On the basis of dose and time responses, these synthetic oestrogens are strong promoters of hepatocarcinogenesis (Yager *et al.*, 1991). Selected studies that support this conclusion are summarized below.

Groups of 12–18 female Sprague-Dawley rats, approximately seven weeks of age, were subjected to a two-thirds partial hepatectomy to induce cell proliferation and initiated by intraperitoneal injection of 20 mg/kg bw NDEA; 24 h later, they were fed a semi-purified diet containing mestranol at a concentration of 0.1 or 0.5 ppm for 9 or 12 months or ethinyloestradiol at 0.5 ppm for nine months. The daily intakes of mestranol were approximately 6 and 30 µg/kg bw or 3–15 times the human contraceptive dose. All survivors were killed at 9 or 12 months, and the livers were evaluated for γ -GT-positive foci and the presence of nodules (adenomas) and carcinomas. By nine months, ethinyloestradiol and mestranol had caused a significant ($p < 0.05$) increase in the number of γ -GT foci but no increase in the incidence of nodules or hepatocellular carcinoma. Mestranol induced a significant, dose-dependent increase in the incidence of hepatocellular carcinomas by 12 months, with incidences of 6/15 animals given NDEA alone, 7/17 animals given NDEA plus mestranol at 0.1 ppm and 11/14 animals given NDEA + 0.5 ppm mestranol. A similar number of foci developed in rats fed 0.5 ppm mestranol and in rats fed a diet containing 50 ppm phenobarbital (Yager *et al.*, 1984).

Ovariectomized Sprague-Dawley rats, 70 days of age, were given a single intraperitoneal injection of 200 mg/kg bw NDEA; beginning on day 80 and every 28 days thereafter for various periods, the rats were treated with subcutaneous implants of Silastic tubing containing a mixture of ethinyloestradiol and cholesterol. The doses of ethinyloestradiol delivered were calculated to be 0, 16, 37, 90 and 230 µg/kg bw per day. After 30 weeks, the proportion of the liver volume occupied by γ -GT-positive, altered hepatic foci showed a linear increase with dose. The increase was statistically significant at 90 and 230 µg/kg bw per day. In initiated rats treated with ethinyloestradiol at 90 µg/kg bw per day, the incidences of hepatic tumours (adenomas + carcinomas) were significantly greater ($p < 0.05$) than in NDEA-initiated controls with cholesterol implants after 30, 40 and 60 weeks of promotion (Campen *et al.*, 1990).

Female Sprague-Dawley rat pups, five days of age, were initiated by an intraperitoneal injection of 10 mg/kg bw NDEA or received no treatment. At weaning, groups of 8–12 rats were fed a semi-synthetic basal diet (controls) or basal diet containing mestranol at a concentration of 0.02 or 0.2 mg/kg (0.02 and 0.2 ppm, respectively) for eight months. When administered alone, mestranol did not induce the appearance of placental glutathione *S*-transferase-positive foci; however, in NDEA-initiated rats, mestranol at a concentration of 0.2 ppm significantly increased ($p < 0.05$) the percentage of the liver volume occupied by foci over that in NDEA-initiated rats fed basal diet. No increase was observed at 0.02 ppm (Dragan *et al.*, 1996).

Three studies have been conducted to determine whether ethinyloestradiol and mestranol initiate carcinogenesis in the liver.

Female Sprague-Dawley rats fed a semi-purified diet underwent a two-thirds hepatectomy and 24 h later, at the peak of regenerative DNA synthesis, groups of 10 rats were treated by gavage with corn oil or mestranol at a dose of 100 or 500 mg/kg bw. A positive control group was injected intraperitoneally with 10 mg/kg bw NDEA. After another 24 h, the rats were transferred to a diet containing 0.05% phenobarbital to promote any hepatocytes that had been initiated. The rats were killed four months later and their livers analysed for γ -GT-positive foci. NDEA initiation caused a more than 10-fold increase in the number of foci/cm², but the number was not significantly increased in rats treated with 100 mg/kg bw mestranol. While there was an approximately fivefold increase in the number of foci in the group fed 500 mg/kg bw, the effect was not statistically significant (Yager & Fifield, 1982).

Male Fischer 344 rats weighing 130–165 g were given various oestrogens and progestogens by intraperitoneal injection approximately 18 h after a two-thirds hepatectomy. Positive controls were treated with *N*-nitrosomorpholine. Two weeks later, the rats were given 0.02% 2-acetylaminofluorene in the diet for two weeks and carbon tetrachloride by gavage at the end of the first week. The animals were then killed, and the numbers of γ -GT-positive foci were determined in 9–15 rats per group. Ethinyloestradiol at a dose of 0.05 mg/kg bw did not increase the number of γ -GT-positive foci over that in controls (Schuppler *et al.*, 1983).

Groups of 12 female Sprague-Dawley rats weighing 140–160 g were fed a semi-purified diet containing ethinyloestradiol at a concentration of 10 ppm for six weeks and then returned to basal diet; controls received basal diet alone. On day 7, all rats were given a two-thirds hepatectomy to increase cell proliferation. After one week on basal diet (week 6–7), the rats were given 0.02% 2-acetylaminofluorene in the diet for two weeks with carbon tetrachloride by gavage at the end of the first week to induce regenerative growth and rapid growth of any initiated foci. The rats were then killed and the numbers of γ -GT-positive foci determined. Ethinyloestradiol caused a significant ($p < 0.01$) fourfold increase in the number of foci/cm² and a sixfold increase in focal area as a percentage of liver volume (Ghia & Mereto, 1989). [The Working Group noted that ethinyloestradiol was administered for five weeks as opposed to a single treatment, as in the previous two studies.]

Prostate models

Ethinyloestradiol has been used in experimental models of prostate cancer to cause reversible atrophy of the prostate. When treatment is withdrawn, the prostate undergoes regrowth and DNA synthesis, setting the stage for initiation by chemical carcinogens. Shirai *et al.* (1986, 1990), Takai *et al.* (1991) and Mori *et al.* (1996) used this protocol. [The Working Group was aware of these studies but did not consider them relevant for evaluating the carcinogenicity of ethinyloestradiol or combinations containing it.]

Kidney models

Rat: Groups of 19–27 male Fischer 344 rats, six weeks of age, were fed diets containing 0.05% *N*-nitrosobis(2-hydroxypropyl)amine (NDHPA), 0.1% *N*-nitrosoethyl-*N*-hydroxyethylamine (NEHEA), 0.03% *N*-nitrosopiperidine (NPip), 0.02% 2-acetylaminofluorene or 0.5% *N*-nitrosobutyl-*N*-(4-hydroxybutyl)amine (NBHBA) for two weeks, followed by 0.001% (10 ppm) ethinyloestradiol for 49 weeks. At that time, ethinyloestradiol was found to have enhanced the incidences of liver hyperplastic nodules in rats initiated with NDHPA, NEHEA, 2-acetylaminofluorene or NPip and to have enhanced the incidence of hepatocellular carcinoma in rats initiated with NEHEA compared with controls; this nitrosamine also enhanced the incidence of kidney adenomas and renal-cell carcinomas. Tumorigenesis was inhibited in the lungs and urinary bladder of rats initiated with NDHPA or NBHBA. Ethinyloestradiol alone had no tumorigenic effect (Shirai *et al.*, 1987).

Hamster: Syrian golden hamsters, five weeks of age, were separated into groups of 30 animals that received four weekly subcutaneous injections of 10 mg/kg bw *N*-nitrosobis(2-oxopropyl)amine (NBOPA) to initiate renal tumorigenesis. These groups then received either control diet or a diet containing 1 ppm ethinyloestradiol for 27 weeks. An additional group of animals was fed the diet containing ethinyloestradiol. Ethinyloestradiol alone did not cause renal tumours or dysplasia. Initiation with NBOPA alone caused the appearance of nephroblastoma in 1/21 animals and 469 dysplastic tubules. Ethinyloestradiol increased the incidence of renal tumours in NBOPA-initiated animals to 4/27 (adenomas) compared with 1/21 (a nephroblastoma) and significantly ($p < 0.001$) increased the number of dysplastic tubules (1602 compared with 469) (Mitsumori *et al.*, 1994).

The results of previous and new studies on oestrogens in mice and rats are summarized in Tables 34 and 35.

3.3 Progestogens used in combined oral contraceptives

3.3.1 Studies reviewed previously

Mouse

Chlormadinone acetate tested by oral administration to mice slightly increased the incidence of benign liver tumours in treated males.

Oral administration of ethynodiol diacetate to mice increased the incidence of benign liver tumours in males and increased the incidence of mammary tumours in castrated males.

Lynoestrenol increased the incidence of benign liver tumours in males and that of malignant mammary tumours in females.

Megestrol acetate increased the incidence of malignant mammary tumours in females.

Norethisterone acetate increased the incidence of benign liver tumours in males.

Norethisterone increased the incidences of benign liver tumours in males and of pituitary adenomas in females.

Oral administration of norethynodrel increased the incidences of pituitary adenomas in animals of each sex, of mammary tumours in castrated males and of malignant mammary tumours in females.

Table 34. Effects of ethinyloestradiol and mestranol alone and with known carcinogens on tumour incidence in mice

Oestrogen	Pituitary adenoma		Malignant mammary tumours		Uterine tumours	Vaginal/cervical tumours	Liver		
	Male	Female	Male	Female			Adenoma		Foci (females)
							Male	Female	
Ethinyloestradiol	+	+	+	+	+	+	+		
Mestranol	+	+	+	+			-	-	
Ethinyloestradiol + <i>N</i> -nitrosodiethylamine									
Ethinyloestradiol + <i>N</i> -nitrosodiethylamine + unleaded gasoline								Protective +	

+, increased tumour incidence; -, no effect

Table 35. Effects of ethinylloestradiol and mestranol alone and with known carcinogens on tumour incidence in rats

Oestrogen	Pituitary adenoma (females)	Malignant mammary tumours (females)	Liver					Kidney	
			Adenoma		Carcinoma		Foci (females)	Adenoma (males)	Carcinoma (females)
			Male	Female	Male	Female			
Ethinylloestradiol	+	+	+	+		+	+		
Mestranol		+					+/-	+	
Ethinylloestradiol + <i>N</i> -nitrosoethyl- <i>N</i> -hydroxyethylamine						+		+	+
Ethinylloestradiol + <i>N</i> -nitrosodiethylamine			+	+	+	+	+	+ ^a	
Mestranol + <i>N</i> -nitrosodiethylamine			+	+	+	+	+	+	

+, increased tumour incidence; -, no effect; +/-, slightly increased tumour incidence

^a In one of three studies, ethinylloestradiol initiated hepatocarcinogenesis

After oral administration of norgestrel to mice, no increase in tumour incidence was observed.

Rat

In rats, oral administration of chlormadinone acetate, megestrol acetate or norgestrel did not increase the incidence of any tumour type.

Ethinodiol diacetate, tested by oral administration to rats, increased the incidence of benign mammary tumours in males.

Lynoestrenol slightly increased the incidence of malignant mammary tumours in females.

Norethisterone increased the incidence of benign liver tumours in males and caused small increases in the incidences of benign and malignant mammary tumours in males and of malignant mammary tumours in females.

Norethynodrel increased the incidences of benign and malignant liver-cell tumours, pituitary adenomas and benign and malignant mammary tumours in males and increased the incidence of benign liver tumours in females.

3.3.2 *New studies*

(a) *Oral administration*

Mouse

Schuppler and Gunzel (1979) summarized data from the study of the Committee on Safety of Medicines (1972) in the United Kingdom and from additional studies on the hepatocarcinogenicity of the progestogens, norgestrel, norethisterone acetate, norethisterone, chlormadinone acetate, ethynodiol diacetate, norethynodrel, megestrol acetate and lynoestrenol, in mice. Increased incidences of liver tumours were detected in groups of 40–80 male CF-LP mice treated with norethisterone acetate, norethisterone, chlormadinone acetate or ethynodiol diacetate and in groups of 40–80 female CF-LP mice treated orally with norethynodrel for 20 months, but the increases were not significant at the 5% level. It was also reported that megestrol acetate given orally at up to 400 times the human contraceptive dose caused a statistically significant increase in the incidence of hepatocellular adenoma in females, from approximately 1% (25 mice) to 5% (73 mice) ($p < 0.05$). Groups of 120 male and female mice [strain not indicated] were treated orally with lynoestrenol at doses up to 400 times the human contraceptive dose for 20 months. The incidence of hepatocellular adenomas was significantly ($p < 0.05$) increased (from approximately 1 to 8%) in males. The incidences induced by megestrol acetate and lynoestrenol were given only as the average for three dose groups, making it impossible to determine a dose–response relationship. There were no statistically significant effects on liver tumour incidence in males or females treated orally with dl-norgestrel alone for 20 months (Schuppler & Gunzel, 1979). [The Working Group noted discrepancies in the numbers of animals and tumour incidences in these two reports but was unable to resolve the differences in the absence of the original data.]

Groups of 40 male and 40 female C57BL/10J mice, seven weeks of age, were fed a diet containing cyproterone acetate obtained by grinding 50-mg tablets of Androcur™ and mixing the powder into the diet at a concentration of 800 mg/kg (ppm) (calculated intake, 125 mg/kg bw per day) for 104 weeks. A control group consisted of eight males and eight females. Cyproterone acetate increased the mortality rate in both males and females after 40 weeks on test: no females survived past 97 weeks, and only four males survived to 104 weeks. The weight of the liver was increased in animals of each sex, and the increase in males was in excess of 100%. In addition, weight gain was reduced such that, at the end of a separate 13-week treatment period, the cyproterone acetate-treated mice weighed 33% less than controls. The causes of death were uterine enlargement in female mice and neoplastic diseases in males. The liver tumour incidences are shown in Table 36. Overall, hepatocellular tumours developed in 44% of the males and 22% of the females. In addition, 85% of the animals developed adenomatous polyps of the pyloric antrum and pancreatic islet hyperplasia (Tucker & Jones, 1996; Tucker *et al.*, 1996). [The Working Group noted that this study has been criticized since the dose of cyproterone acetate administered clearly exceeded the maximum tolerated dose (Schauer *et al.*, 1996).]

Table 36. Effects of cyproterone acetate (CPA) on liver tumour incidence in C57BL/10J mice

Liver tumour	Males		Females	
	Control	CPA	Control	CPA
Hepatocellular adenoma	0/8	7/39	0/8	2/37
Hepatocellular carcinoma	0/8	12/39	0/8	8/37

From Tucker & Jones (1996); Tucker *et al.* (1996)

Rat

Schuppler and Gunzel (1979) summarized data from the study of the Committee on Safety of Medicines (1972) on the hepatocarcinogenicity in rats of a number of progestogens. Rats [strain unspecified] were treated orally with the progestogens for two years at doses up to 400 times the human contraceptive dose. Table 37 summarizes the results presented in their paper, which indicate statistically significant increases. Cyproterone acetate at doses 200–400 times the human contraceptive dose did not increase the incidence of hepatocellular adenomas in another study in this report. In a further study, groups of 35 male and 35 female rats were treated orally with cyproterone acetate at doses of 250, 1250 or 6250 times the human contraceptive dose and were observed for 20 months. In males, a significant ($p < 0.01$) increase in the incidence of liver adenomas occurred only at 6250 times the human contraceptive dose, while in females a significant ($p < 0.01$) increase was observed at both 1250 and 6250 that dose (Schuppler *et al.*, 1977; Schuppler & Gunzel, 1979).

Table 37. Effects of various progestogens on the incidence of hepatocellular adenomas in rats

Progestogen	Males	Females
Norgestrel	–	–
Norethisterone	+	–
Chlormadinone acetate	–	–
Ethinodiol diacetate	–	–
Norethynodrel	+	+
Lynoeestrenol	–	–
Megestrol acetate	–	–

From Committee on Safety of Medicines (1972)

Albino Sprague-Dawley-derived rats were fed diets containing 7.5 or 75 ppm norethisterone acetate, which provided intakes approximately 10 and 100 times the human contraceptive dose. The actual progestogen intake was stated to be 0.303 mg/kg bw for males and 0.397 mg/kg bw for females at the low dose and 3.18 mg/kg bw for males and 4.15 mg/kg bw for females at the high dose. Survival over the two-year study was greater in the treated (22%) than in control (10%) rats. Dose-related effects were seen in liver enlargement, numbers of altered hepatic foci and liver neoplastic nodules (adenomas or regenerative nodules) and the incidence of uterine polyps [details not reported]. No statistically significant increase in the incidence of malignant tumours was observed in the liver or other organs (Schardein, 1980).

Male Fischer 344 rats weighing 130–150 g were subjected to a partial hepatectomy 18 h before treatment with a microcrystalline suspension of cyproterone acetate (purity analytically confirmed) in saline as a single intraperitoneal injection of 100 mg/kg bw. Thirteen days later, the rats were fed a diet containing 0.02% 2-acetylaminofluorene to inhibit normal hepatocyte growth, and seven days later, the rats were given 2 mL/kg bw carbon tetrachloride to cause hepatocyte necrosis and stimulate regenerative growth. One week later, the rats were killed and their livers analysed for γ -GT-positive foci. Cyproterone acetate did not significantly increase the number of γ -GT-positive foci over control values (Schuppler *et al.*, 1983). [The Working Group noted the use of a single dose and only male rats.]

The tumour initiating activity of cyproterone acetate was tested in groups of six female Sprague-Dawley rats, 22 days of age at the start of treatment, given 0 (vehicle control), 25, 50 or 100 mg/kg bw orally in olive oil on five consecutive days. One week after the last treatment, the rats were given 10 mg/kg bw Clophen A50 (a technical mixture of polychlorinated biphenyls) as a tumour promoter twice weekly for 11 weeks. One group of four animals was untreated. The livers were analysed for the presence of ATPase-deficient and γ -GT-positive foci. The numbers and area of these foci were significantly increased in a dose-dependent manner by cyproterone acetate (Deml *et al.*, 1993).

Dog

Groups of 16 young pure-bred beagle bitches received lynoestrenol orally in tablet form at a dose representing 10, 50 and 125 times the human contraceptive dose daily for 364 weeks; controls received a placebo tablet. The results are summarized in Table 38. A biphasic dose–response effect on mammary tumorigenesis was seen: at the low dose, lynoestrenol appeared to protect against the development of mammary tumours, but at the intermediate and high doses, it was associated with increased incidences of mammary nodules and carcinomas [statistics not specified] (Misdorp, 1991).

Table 38. Effects of lynoestrol on mammary tumour incidence in beagle bitches

Treatment	Nodule incidence	Nodule latency (weeks)	Carcinoma incidence
Control	5/16	323	1/16
10 × HCD	0/16*		[0]
50 × HCD	16/16	191**	3/16 [NR]
125 × HCD	16/16	152**	7/16 [NR]

From Misdorp (1991); HCD, human contraceptive dose; [NR], statistical analysis not reported

*Significantly lower than in other groups ($p < 0.05$)

**Significantly earlier than in controls ($p < 0.05$)

In a study to determine the six-month toxicity of the progestogen STS 557, levonorgestrel was administered as control to four female and four male beagles, 7–12 months of age, at a dose of 1 mg/kg bw orally seven times a week for six months. Mammary hyperplasia but no nodules or malignant tumours was observed (Hoffmann *et al.*, 1983). [The Working Group noted the short duration of the study.]

(b) *Administration with known carcinogens*

Mouse

Groups of 20 female B6AF₁ mice, 12 weeks of age, received a silk thread impregnated with 3-methylcholanthrene inserted into the cervical canal and passed through the uterine wall; a control group received unimpregnated silk thread. Pellets containing 15 mg per mouse norethynodrel and 0.5 mg per mouse mestranol, alone and in combination, were then implanted subcutaneously and were renewed every three weeks for a total of 15 weeks. No tumours developed in the mice that did not receive 3-methylcholanthrene, but the steroids caused various histopathological changes in the mucosa of the cervix, uterus and vagina. Norethynodrel alone promoted the incidence of uterine tumours (11/14 compared with 5/35 in controls) but not of cervical or vaginal tumours (Blanzat-Reboud & Russfield, 1969).

Rat

Female Sprague-Dawley rats, seven weeks of age, were initiated with NDEA 24 h after partial hepatectomy; 24 h later, they were fed a diet containing norethynodrel, providing intakes of 0.5–0.75 mg/kg bw per day for nine months. After four months, a statistically significant ($p < 0.05$), sixfold increase in the number of γ -GT-positive, altered hepatic foci was observed in comparison with rats given NDEA alone. At nine months, the number of foci was reduced and significantly greater than with NDEA alone only when one norethynodrel-treated rat with a large number of foci was deleted from the analysis. No significant increase in the incidence of nodules or carcinomas was observed after nine months (Yager & Yager, 1980).

Male Fischer 344 rats, weighing 130–150 g, were subjected to a partial hepatectomy and 18 h later were given norethynodrel or norethisterone acetate (purity confirmed analytically) by intraperitoneal injection of 100 mg/kg bw as a microcrystalline suspension in saline; 13 days later, the rats were fed a diet containing 0.02% acetylaminofluorene to inhibit normal hepatocyte growth, and seven days later the rats were given 2 mL/kg bw carbon tetrachloride to cause hepatocyte necrosis and stimulate regenerative growth. One week later, the rats were killed and their livers were analysed for γ -GT-positive foci. Neither norethynodrel nor norethisterone acetate significantly increased the number of γ -GT-positive foci over control values (Schuppler *et al.*, 1983).

Hamster: Groups of 30 Syrian golden hamsters, five weeks of age, received four weekly subcutaneous injections of *N*-nitrosobis(2-oxypropyl)amine (NBOPA) at a dose of 10 mg/kg bw to initiate renal tumorigenesis and then received either control diet or a diet containing 10 mg/kg diet (ppm) levonorgestrel for 27 weeks. A third group of animals was not treated with the nitrosamine but was fed the diet containing levonorgestrel. Levonorgestrel alone did not cause renal tumours or dysplasia. Initiation with NBOPA caused nephroblastoma in 1/21 animals and 469 dysplastic tubules. Levonorgestrel did not significantly enhance the incidence of renal tumours in initiated animals (2/27 nephroblastomas and 2/27 renal adenomas) or increase the total number of dysplastic tubules (747) (Mitsumori *et al.*, 1994).

The results of previous and new studies on progestogens are summarized in Tables 39–41.