4. Other Data Relevant to an Evaluation of Carcinogenicity and its Mechanisms

4.1 Absorption, distribution, metabolism and excretion

The disposition of various formulations of oral contraceptives used in humans differs. In general, both the oestrogenic and progestogenic compounds in combined oral contraceptives are absorbed by the gut and metabolized largely in the liver. A fraction of the absorbed dose of ethinyloestradiol and several progestogens is excreted in the bile during

Table 39. Effects of various progestogens alone and with a known carcinogen on tumour incidence in mice

Progestogen	Pituitary adenoma		Mammary tumours		Uterine tumours	Vaginal/ cervical tumours	Liver			
	Male	Female	Benign (males)	Malignant (females)			Adenoma		Carcinoma	
							Male	Female	Male	Female
Chlormadinone acetate							+/–			
Cyproterone acetate							+[a]	+/–[a]	+[a]	+[a]
Ethynodiol diacetate			c				+/–			
Lynoestrenol				+			+			
Megestrol acetate				+				+		
Norethisterone acetate							+/–			
Norethisterone		+					+/–			
Norethynodrel	+	+	c	+				+/–		
Norethynodrel + 3-methyl-cholanthrene					+	–				

+, increased tumour incidence; +/–, slightly increased tumour incidence; –, no effect; c, increased incidence in castrated males

[a] Dose exceeded the maximum tolerated daily dose

Table 40. Effects of various progestogens alone and with a known carcinogen on tumour incidence in rats

Progestogen	Pituitary adenoma (males)	Mammary tumours			Liver				
		Benign (males)	Malignant		Adenoma		Carcinoma (males)	Foci	
			Male	Female	Male	Female		Male	Female
Cyproterone acetate					+[a]	+[a]			+[b]
Ethynodiol diacetate		+							
Lynoestrenol				+/–					
Norethisterone acetate					+	+		+	+ or –[c]
Norethisterone		+/–	+/–	+/–	+				
Norethynodrel	+	+	+		+	+	+		–[c]
Norethynodrel + *N*-nitrosodiethylamine									+

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

[a] Liver adenomas detected only at high doses

[b] Tested for initiating activity; the results were positive in one study in which it was administered for five days and negative when administered as a single dose

[c] Tested as a single dose for initiating activity

Table 41. Effects of various progestogens on mammary tumour incidence in bitches

Progestogen	Benign	Malignant
Chlormadinone acetate	+	+
Lynoestrenol	+[a]	+[a]
Megestrol acetate	+	+

+, increased tumour incidence

[a] In this study, lynoestrenol had a biphasic effect, with protection at the low dose (10 times the human contraceptive dose) and enhancement at 50 and 125 times the human contraceptive dose.

its first transit through the liver. Although some of these compounds are partially re-absorbed via the enterohepatic circulation, a fraction may be lost in this 'first pass', reducing the overall bioavailability. The absorption rates are usually rapid, peak serum values being observed between 0.5 and 4 h after intake. Serum concentrations rise faster with multiple treatments than single doses and achieve higher steady-state levels, which are still punctuated by rises after each daily dose. The rise in steady-state levels with multiple doses may reflect the inhibitory effect of both oestrogens and progestogens on cytochrome P450 metabolic enzyme activities. Alternatively, oestrogens may induce the production of sex hormone-binding globulin, which may increase the capacity of the blood to carry progestogens. Binding of progestogen to the sex hormone-binding globulin may displace oestrogens and androgens, which may then cause adverse androgenic side-effects and alter serum lipid concentrations. The metabolism of progestogens and ethinyloestradiol typically involves oxidative modifications. In some cases, metabolism converts an inactive pro-drug into a hormonally active compound. Oxidized metabolites are typically conjugated as glucuronides or sulfates, and most are eliminated rapidly, with half-lives of 8–24 h.

Kopera (1985) reviewed the drug interactions associated with administration of progestogens to patients receiving other medications. Progestogens adversely affect the metabolism of various drugs and, in turn, the metabolism of progestogens is affected by the other drugs. These effects occur presumably as a consequence of effects on the induction of metabolic enzymes or on competition for metabolic pathways or for binding to serum carrier proteins.

Thomas *et al*. (1993) studied a single menstrual cycle in 25 pre-menopausal women who smoked five or more cigarettes per day and 21 non-smoking women to compare the plasma concentrations of luteinizing hormone, follicle-stimulating hormone, oestradiol, progesterone, testosterone, androstenedione, dehydroepiandrosterone sulfate and sex hormone-binding globulin and urinary excretion of oestradiol, oestriol and oestrone. No significant differences were found between the two groups for these parameters or in the lengths of the follicular and luteal phases.

Kuhnz and Löfberg (1995) evaluated the ratio of 6β-hydroxycortisol to cortisol excreted in urine as a measure of drug metabolizing activity. Groups of 12–15 women received combined oral contraceptives containing levonorgestrel, gestodene or cyproterone acetate in combination with ethinyloestradiol, or levonorgestrel or gestodene alone. Little or no difference in the ratio was observed between groups.

Coenen *et al*. (1996) gave groups of 22 women oral monophasic combined contraceptives containing 35, 30, 30 or 20 μg ethinyloestradiol with 250 μg norgestimate, 75 μg gestodene, 150 μg desogestrel or 150 μg desogestrel, respectively. Each woman received a dose once a day for 21 days of a 28-day cycle for six cycles. All of the steroidal serum parameters tested (total testosterone, free testosterone, dihydrotestosterone, androstenedione) were significantly decreased, and the concentrations of the steroid-binding proteins, sex hormone-binding globulin and cortisol-binding globulin were significantly increased, irrespective of the oral contraceptive preparation used. Differences between the groups were observed only in dehydroepiandrosterone sulfate and cortisol-binding globulin.

4.1.1 *Ethinyloestradiol*

(*a*) *Humans*

Goldzieher and Brody (1990) reviewed information about the pharmacokinetics of ethinyloestradiol and mestranol given in a dose of 35 and 50 μg, respectively, in combination with 1 mg norethisterone. A group of 24 women received ethinyloestradiol and 27 women received mestranol. Serum ethinyloestradiol concentrations were measured after treatment with either oestrogen. Both treatments produced equal average serum concentrations of about 175 pg/mL, but there was wide inter-individual variation. The maximal serum concentrations were achieved in about 1–2 h, and the half-life for elimination ranged from 13 to 27 h. Intra-individual variation in the plasma concentration of ethinyloestradiol derived from mestranol did not differ significantly from that observed after ethinyloestradiol treatment. The oral bioavailability of ethinyloestradiol was only 38–48%. The authors also reviewed their earlier studies of patterns of urinary conjugates, glucuronides and sulfates in women from Nigeria, Sri Lanka and the United States after oral administration of radiolabelled ethinyloestradiol. The proportions of glucuronides and sulfates were about 70 and 18%, respectively, in each population; however, the Nigerian women had the lowest concentrations of oxidative metabolites and the American women the highest. [The basis for this diversity, whether genetic, nutritional or environmental, was unclear.]

Hümpel *et al*. (1990) obtained serum samples from a group of 30 women during one cycle of a combined oral contraceptive containing ethinyloestradiol and desogestrel and from a group of 39 women taking ethinyloestradiol and gestodene. The mean serum concentrations were 186–226 nmol/L sex hormone-binding globulin, 89–93 mg/L cortisol-binding globulin and 280–281 μg/L cortisol. The serum concentrations of ethinyloestradiol reached mean maximum levels of 106–129 pg/mL 1.6–1.8 h after pill intake.

Kuhnz *et al*. (1990a) compared the pharmacokinetics of ethinyloestradiol given as a single dose in combination with either gestodene or desogestrel to 18 women. In contrast to previous reports that the bioavailability of ethinyloestradiol differed according to the associated progestogen, this study showed no significant difference. The maximum concentration of ethinyloestradiol was found 1.9 h after ingestion and reached 101 and 104 pg/mL for the two combinations, respectively. The values for maximum concentrations and for the integral of the serum concentration over time (area under the concentration curve) differed between individuals, but, for each individual, the concentration of ethinyloestradiol reached with the two contraceptives was usually about the same.

(*b*) *Experimental systems*

Standeven *et al*. (1990) studied the metabolism of ethinyloestradiol in primary cultures of rat hepatocytes. At 4, 24 or 48 h after establishment in culture, the cells maintained their ability to metabolize up to 90% of ethinyloestradiol substrate (4 nmol/L or 2 μmol/L) to polar conjugates during a 4-h incubation. The metabolites formed were reported to differ both quantitatively and qualitatively from those formed in rats *in vivo*.

The major pathway of ethinyloestradiol metabolism in humans and animals is 2-hydroxylation, which is presumably catalysed by the 3A4 isoform of cytochrome P450 (Guengerich, 1988; Yager & Liehr, 1996). Like catechols of oestrone and oestradiol, hydroxylated metabolites of ethinyloestradiol can also undergo redox cycling and damage DNA (Yager & Liehr, 1996).

4.1.2 *Mestranol*

(*a*) *Humans*

The pharmacokinetics of mestranol has been investigated (Goldzieher & Brody, 1990) and reviewed (Bolt, 1979; Kuhl, 1990).

Mestranol is a pro-drug that binds poorly to the oestrogen receptor until it is demethylated in the gastrointestinal tract to its active form, ethinyloestradiol; 54% of mestranol is converted to ethinyloestradiol (Bolt & Bolt, 1974). Since the demethylation is not complete, more mestranol than ethinyloestradiol must be administered to achieve the same effect. The pharmacokinetics of mestranol corresponds to that of ethinyloestradiol, except that the peak concentrations are lower. Since mestranol is more lipophilic than ethinyloestradiol, it can be stored in fatty tissues (Bolt, 1979).

In a study by Goldzieher and Brody (1990), 24 women received ethinyloestradiol and 27 were given mestranol, both in combination with norethisterone. The bioavailability and maximum concentration of mestranol were about 30% lower than those of ethinyloestradiol. A 50-μg oral dose of mestranol was bioequivalent to a 35-μg dose of ethinyloestradiol, both administered in combination with 1 mg norethisterone. Administration of 50 μg mestranol resulted in a mean maximum concentration of ethinyloestradiol of 175 pg/mL at 1.9 h. Intra-individual differences in the plasma concentration of ethinyloestradiol over 24 h were large, however, when the effects of single doses were compared in the same individual at different times. The metabolites of mestranol found

in urine are, apart from ethinyloestradiol, 2-hydroxyethinyloestradiol, 2-methoxyethinyloestradiol and 2-hydroxyethinyloestradiol-3-methyl ether (reviewed by Bolt, 1979).

(*b*) *Experimental systems*

Studies in rats have shown that metabolites of mestranol undergo enterohepatic circulation, which may be affected by antibiotics such as neomycin (Brewster *et al*., 1977). Further metabolism of demethylated mestranol is species-specific; for example, 2-hydroxylation occurs in rats and D-homo-annulation in rabbits and guinea-pigs (Abdel-Aziz & Williams, 1969; Ball *et al*., 1973).

4.1.3 *Chlormadinone acetate*

(*a*) *Humans*

The pharmacokinetics of chlormadinone acetate has been reviewed by Kuhl (1990) and in previous *IARC Monographs* (IARC, 1974, 1979); no recent data are available in humans, probably because there has been no or limited use since the early 1970s.

After intravenous injection of radiolabelled chlormadinone acetate, the steroid and its metabolites have an initial rapid half-life of 2.4 h, followed by a slow half-life of 80.1 h. The mean metabolic clearance rate is 126 L/day for chlormadinone acetate and 42.6 L/day for chlormadinone acetate and its metabolites. The long half-life and slow elimination rate are probably due to accumulation of the drug in fat tissue (Dugwekar *et al*., 1973).

(*b*) *Experimental systems*

The major metabolites of chlormadinone acetate are 2α-hydroxychlormadinone acetate and 3β-hydroxychlormadinone acetate. Incubation of chlormadinone acetate with human or rat liver microsomes produces mainly the 3β-hydroxy metabolite. In contrast, incubation with microsomes from phenobarbital-treated rats produces the 2α-hydroxy metabolite, indicating that the metabolite pattern is dependent on the hepatic monooxygenase state (Handy *et al*., 1974).

4.1.4 *Cyproterone acetate*

(*a*) *Humans*

A group of eight young women were treated with a single oral dose of 100 mg cyproterone acetate followed by a single intramuscular dose of 300 mg four weeks later, and the plasma concentration of both parent compound and the 15β-hydroxy metabolite were quantified in seven of the women. The bioavailability of cyproterone acetate after oral administration was about 88%; the mean maximum serum concentration reached 255 ng/mL between 2 and 3 h, and thereafter decreased biphasically, reaching a terminal half-life of about 3.6 days. After intramuscular injection, the serum concentration reached 191 ng/mL after two to three days and then declined, with a half-life of about 4.3 days. The serum concentrations of the 15β-hydroxy metabolite exceeded those of the parent compound 6 h after oral administration and four days after intramuscular injection.

Thereafter, the concentration of the 15β-hydroxy metabolite decreased at a rate parallel to that of cyproterone acetate, indicating that the formation of this metabolite was the rate-limiting metabolic step (Huber *et al*., 1988).

A group of 15 women was treated with a single oral dose of 2.0 mg cyproterone acetate plus 0.035 mg ethinyloestradiol. After one week, three cycles of multiple treatments were started with the same preparation. After the single dose, the maximum concentration of cyproterone acetate was 15.2 ng/mL, which decreased biphasically with half-lives of 0.8 and 54 h, respectively; 3.5% of the dose was free, while 96.5% was bound to serum proteins. During the multiple treatment cycles, a twofold higher accumulation of cyproterone acetate was observed, and its half-life increased to 78 h (Kuhnz *et al*., 1993a).

In a study to determine the bioequivalence of one 100-mg and two 50-mg tablets and to compare two analytical methods for cyproterone acetate, 36 young men received one 100-mg dose followed three weeks later by two 50-mg tablets. The mean maximum concentrations of cyproterone acetate in serum were 200–260 ng/mL 2–3 h after dosing, followed by a second peak between 6 and 12 h. Thereafter, the concentrations decreased biphasically until 120 h after dosing, reaching a mean half-life of about 50 h (Baumann *et al*., 1996).

(*b*) *Experimental systems*

No data were available to the Working Group.

4.1.5 *Desogestrel*

(*a*) *Humans*

McClamrock and Adashi (1993) reported that desogestrel is metabolized rapidly and completely in the liver and gut wall. It is metabolized to 3-keto-desogestrel, which mediates its progestogenic effects, and it is not metabolized further to another progestogen. The serum concentrations of 3-keto-desogestrel reached maximum levels within 2–3 h after oral administration of desogestrel and were subsequently cleared with a half-life of 12–24 h. In a review (Stone, 1995), it was reported that desogestrel reaches a steady-state serum concentration within 8–10 days. In serum, about 5% of desogestrel circulates freely, while 65% is bound to albumin and 30% to sex hormone-binding globulin.

Madden *et al*. (1990) studied the metabolism of desogestrel in microsomes from six human livers *in vitro*. The main metabolite formed was 3-keto-desogestrel; 3α-hydroxy-desogestrel and 3β-hydroxydesogestrel were also detected. The metabolism of desogestrel was inhibited by 50% by primaquine at a concentration of 30 μmol/L, but not by levonorgestrel at 250 μmol/L.

Nineteen women were given three cycles of a triphasic oral contraceptive with combinations of desogestrel and ethinyloestradiol at doses of 50 and 35 μg for the first seven days, 100 and 30 μg for days 8–14 and 150 and 30 μg for days 15–21, respectively, followed by seven days without hormone. Multiple blood samples were taken from the women throughout this interval, and serum concentrations of 3-keto-desogestrel, ethinyl-

oestradiol and sex hormone-binding globulin were determined, together with the elimination half-life and dose proportionality. The concentration of 3-keto-desogestrel reached steady-state level at each desogestrel dose, and the pharmacokinetics was proportional to dose. The concentration of ethinyloestradiol also reached a steady state, and the pharmacokinetics was constant thereafter. The concentration of sex hormone-binding globulin was significantly increased between days 1 and 7 of the cycle but not between days 7, 14 and 21 (Archer *et al*., 1994).

(*b*) *Experimental systems*

No data were available to the Working Group.

4.1.6 *Gestodene*

(*a*) *Humans*

Gestodene is an active progestogen that has an oral bioavailability of almost 100% and shows pharmacokinetics linear to dose. The serum concentrations are four times higher after multiple treatment cycles than after one cycle, and the area under the concentration curve increases by five- to eightfold after multiple cycles of gestodene plus ethinyloestradiol. Gestodene is metabolized primarily in the liver by P450 CYP 3A4, and it is a strong inducer of this enzyme. Although ethinyloestradiol is also metabolized by CYP 3A4, gestodene does not appear to inhibit its metabolism. Known metabolites of gestodene include dihydrogestodene, 3,5-tetrahydrogestodene and hydroxygestodene. After a single 75-µg dose of gestodene alone, 64% of the compound was bound to sex hormone-binding globulin in the serum, 34% was bound to albumin and about 1.3% was free. Clearance is dependent on the concentration of free gestodene. The half-life of clearance and elimination is 10–18 h and is higher after multiple doses than after a single dose of gestodene plus ethinyloestradiol. Monophasic preparations typically contain 75 µg gestodene plus 20 or 30 µg ethinyloestradiol, given for 21 days per 28-day cycle. Triphasic preparations contain 50, 70 or 100 µg of gestodene combined with 30, 40 and 30 µg ethinyloestradiol, respectively, in phases administered for weeks 1, 2, and 3 of a four-week cycle. Gestodene does not reduce the oestrogen-induced increases in the concentration of sex hormone-binding globulin and does not affect serum testosterone levels (Shoupe, 1994; Kuhl *et al*., 1995; Wilde & Balfour, 1995). Täuber *et al*. (1990) found that orally administered gestodene is completely absorbed and exhibits dose-linear pharmacokinetics. The maximum serum concentrations reached 1, 3 and 5 ng/mL after single doses of 25, 75 and 125 µg, respectively. Only 0.6% was not bound to protein, while 75% was bound to sex hormone-binding globulin and 24% to albumin.

Kuhnz *et al*. (1990b) studied the binding of gestodene to serum protein in 37 women who had taken a combined oral contraceptive containing gestodene plus ethinyloestradiol for at least three months: 0.6% was free, while 24% was bound to albumin and 75% to sex hormone-binding globulin .

Kuhnz *et al*. (1991) examined the effects of single and multiple administrations of a triphasic combined oral contraceptive containing gestodene and ethinyloestradiol on the

concentrations of ethinyloestradiol and testosterone in 10 women. After a single oral dose of 0.1 mg gestodene plus 0.03 mg ethinyloestradiol, the serum ethinyloestradiol concentration reached 100 pg/mL in about 1.9 h; thereafter, the concentration declined, with a half-life of 11 h. On day 21 of the treatment cycle, the maximum concentrations reached 140 pg/mL 1.6 h after pill intake. In comparison with pretreatment concentrations, those of total and free testosterone were reduced by about 60%.

Kuhnz *et al*. (1993b) treated 14 women with a combined oral contraceptive containing 0.1 mg gestodene plus 0.03 mg ethinyloestradiol as a single dose or for three months as a triphasic regimen. The maximum serum concentrations of gestodene were 4.3 ng/mL after a single dose, 15 ng/mL at the end of the first cycle and 14.4 ng/mL at the end of three cycles, reached 30 min after dosing. A half-life for clearance of 18 h was observed after a single treatment, the volume of distribution being 84 L. Multiple treatments increased the clearance half-life to 20–22 h and reduced the distribution volume to about 18 L. The serum sex hormone-binding globulin concentration increased with multiple treatments, presumably as an effect of ethinyloestradiol; this change in serum protein concentration is thought to account for the observed change in the distribution of gestodene, from 1.3% free, 69% bound to sex hormone-binding globulin and 29% bound to albumin after a single treatment, to 0.6% free, 81% bound to sex hormone-binding globulin and 18% bound to albumin after multiple treatments.

Heuner *et al*. (1995) treated 14 women with a combined oral contraceptive containing 0.1 mg gestodene plus 0.03 mg ethinyloestradiol as a single administration or for three months as a triphasic regimen. The serum concentrations of gestodene, ethinyloestradiol, cortisol-binding globulin, sex hormone-binding globulin and testosterone were followed after the single treatment and through cycles 1 and 3. The serum concentration of ethinyloestradiol reached a peak of about 65 pg/mL by 1.7 h after oral administration; after multiple treatments, the maximum was as high as 90 pg/mL, but the time to reach the maximum concentration was unchanged. The concentration of gestodene reached a maximum of 3.5 ng/mL within 0.7 h after a single dose and 8.7 ng/mL within 0.9 h after multiple doses. The clearance half-time for a single dose of gestodene also increased, from 12.6 h to nearly 20 h. There was a large increase in the concentration with time after multiple treatments. After a single dose, 1.3% of gestodene in serum was unbound, while 30% was bound to albumin and 68% was bound to sex hormone-binding globulin.

(*b*) *Experimental systems*

No data were available to the Working Group.

4.1.7 *Levonorgestrel* (see also the monograph on ‘Hormonal contraceptives, progestogens only’, section 4.1.2)

(*a*) *Humans*

The clinical pharmacokinetics and metabolic effects of levonorgestrel have been reviewed (Fotherby, 1995; Lachnit-Fixson, 1996). Lipid metabolism appears to be largely unaffected by three-phasic administration of levonorgestrel, most studies showing no

significant change in the concentrations of high- or low-density lipoprotein or cholesterol. Effects on carbohydrate metabolism have been described, but the results are not consistent. Since levonorgestrel binds strongly to sex hormone-binding globulin, its pharmacokinetics is affected by the large number of factors that affect this globulin.

Stanczyk and Roy (1990) reviewed the metabolism of levonorgestrel in women treated orally with radioactively labelled compound. Levonorgestrel was found mostly untransformed in serum within 1–2 h after administration, but the concentrations of conjugated metabolites increased progressively between 4 and 24 h after ingestion. Most of the conjugates were sulfates and glucuronides. In addition to the remaining unconjugated levonorgestrel, considerable amounts of unconjugated and sulfate-conjugated forms of 3α,5β-tetrahydrolevonorgestrel were found; smaller quantities of conjugated and unconjugated 3α,5α-tetrahydrolevonorgestrel and 16β-hydroxylevonorgestrel were also identified (Sisenwine *et al.*, 1975a). About 45% of radioactively labelled levonorgestrel was excreted via the urine and about 32% via the faeces. The major urinary metabolites were glucuronides—most abundantly 3α,5β-tetrahydrolevonorgestrel glucuronide—and smaller quantities of sulfates (Sisenwine *et al.*, 1975b).

Carol *et al*. (1992) evaluated the pharmacokinetics of levonorgestrel in groups of 11–20 women given single or multiple treatments with combined oral contraceptive preparations containing 125 μg levonorgestrel plus 30 or 50 μg ethinyloestradiol. The serum concentrations of levonorgestrel reached a maximum of about 4 ng/mL 1–2 h after a single treatment with either preparation. After 21 days of treatment, the peak and sustained concentrations of levonorgestrel were about twice as high as those after a single treatment. The serum concentration of sex hormone-binding globulin increased after treatment with both contraceptives but to a greater extent with the contraceptive containing 50 μg ethinyloestradiol, indicating the important role of the oestrogen in induction of this protein.

Kuhnz *et al*. (1994a) treated 14 women with a combined oral contraceptive containing 0.125 mg levonorgestrel plus 0.03 mg ethinyloestradiol as a single dose or for three months as a triphasic regimen. The serum concentration of free levonorgestrel reached a peak of 0.06–0.08 ng/mL about 1 h after treatment. In contrast, the calculated values of the area under the concentration curve more than doubled, from 0.32 to 0.75–0.77 ng × h/mL, during the first and third multiple treatment cycles. The serum concentrations of cortisol-binding globulin and sex hormone-binding globulin more than doubled after multiple treatments with the contraceptive. After a single dose, 1.4% of the levonorgestrel in serum was free, while 43% was bound to albumin and 55% to sex hormone-binding globulin. After multiple treatments, only 0.9–1.0% levonorgestrel in serum was free and 25–30% was bound to albumin, while the amount bound to sex hormone-binding globulin increased to 69–74%. The concentrations of free and total testosterone decreased from 3 and 460 pg/mL, respectively, before treatment to 1 and 270 pg/mL, respectively, at the end of one treatment cycle, but had increased again to 2 and 420 pg/mL by the first day of the third cycle.

Kuhnz *et al*. (1992) treated groups of eight to nine women with a combined oral contraceptive containing 0.15 mg levonorgestrel plus 0.03 mg ethinyloestradiol as a single

dose or for three months on a monophasic regimen. The peak concentrations of levonorgestrel were found 1 h after single or multiple treatments, but the peak serum concentrations were 3.1 and 5.9 ng/mL, respectively. The area under the concentration curve increased by two- to fourfold for total and free levonorgestrel when a single dose was compared with multiple treatments. The distribution of free, albumin-bound and sex hormone-binding globulin-bound levonorgestrel was similar in women who had received one or multiple treatments, but the serum concentration of the globulin increased significantly after multiple treatments.

(*b*) *Experimental systems*

Kuhnz *et al*. (1995) studied aspects of the pharmacokinetics of levonorgestrel, norgestimate and levonorgestrel-oxime in rats, the last two compounds being pro-drugs of levonorgestrel. The maximum concentration of levonorgestrel was reached about 1 h after treatment and decreased thereafter. In animals treated with norgestimate or levonorgestrel-oxime, the serum concentration of levonorgestrel increased up to about 8 h after treatment and decreased only slightly thereafter up to 24 h after treatment. The total dose ingested, measured as the area under the concentration curve, for levonorgestrel during a 24-h interval was related linearly to the administered dose of each compound.

In a trial of drugs for pregnancy maintenance, Kuhnz and Beier (1994) administered levonorgestrel at a dose of 10–300 μg/day or norgestimate at 30–1000 μg/day subcutaneously to pregnant rats which had been ovariectomized on day 8 of pregnancy. These rats also received a daily dose of 1 μg oestrone. Doses of 300 μg/day of either compound fully maintained pregnancy. In serum samples collected from each animal, the concentration of levonorgestrel increased up to 2–8 h after administration and remained at a plateau thereafter up to 24 h. The area under the concentration curve during the 24-h interval after administration was linearly related to the administered dose of levonorgestrel.

4.1.8 *Megestrol acetate*

(*a*) *Humans*

After administration of megestrol acetate at 160 mg/day to post-menopausal women with advanced breast cancer, the maximum concentration in serum was reached within 2–4 h. Co-administration of megestrol acetate and aminoglutethimide decreased the serum concentration of megestrol acetate by 74% (Lundgren *et al*., 1990).

Megestrol acetate is hydroxylated at various positions of the steroid molecule (Cooper & Kellie, 1968; Lundgren *et al*., 1990). It is metabolized more slowly than progesterone. The 17α-acetoxy group and the 6(7)-double bond are considered to provide resistance to metabolism by liver enzymes (Cooke & Vallance, 1965). The major route of elimination in humans is via the urine. After administration of 4–90 mg radiolabelled megestrol acetate to patients, 56–78% was excreted in the urine and only 7–30% in faeces; 5–8% of that in urine was present as metabolites (Cooper & Kellie, 1968).

(*b*) *Experimental systems*

No data were available to the Working Group.

4.1.9 *Norethisterone* (see also the monograph on 'Hormonal contraceptives, progestogens only, section 4.1.3)

(*a*) *Humans*

Although norethisterone is absorbed almost completely, it undergoes first-pass metabolism, which decreases its bioavailability to an average of 64%. There is wide inter-individual variation in its absorption, which is estimated to be as high as three- to fivefold. Norethisterone is absorbed rapidly, achieving maximum serum concentrations within 1–4 h. After doses of 0.5, 1 and 3 mg, the serum concentrations peaked at 2–5, 5–10 and up to 30 ng/mL, respectively. When given in combination with ethinyloestradiol, norethisterone reaches higher serum levels, which also increase with multiple doses until they reach a steady state at high concentrations. The higher steady-state level has been attributed to a reduced rate of metabolism when norethisterone and ethinyloestradiol are combined. Furthermore, the oestrogen induces sex hormone-binding globulin which binds norethisterone and changes the relative distribution of free and albumin-bound norethisterone. The half-life for elimination is about 8–10 h. Norethisterone is stored in various target organs, and about 22% of the dose accumulates in fat (Kuhl, 1990).

The major metabolites of norethisterone are isomers of 5α-dihydronorethisterone and tetrahydronorethisterone, which are excreted largely as glucuronides. Because of steric hindrance of the bulky ethinyl group at position 17α, only a small percentage of norethisterone metabolites are conjugated at the 17β-hydroxy group. The ethinyl group remains intact in 90% of metabolites (Kuhl, 1990; Shenfield & Griffin, 1991).

(*b*) *Experimental systems*

No data were available to the Working Group.

4.1.10 *Lynoestrenol, ethynodiol diacetate and norethynodrel*

(*a*) *Humans*

Lynoestrenol, ethynodiol diacetate and norethynodrel are pro-drugs of norethisterone. Both lynoestrenol and norethynodrel are converted into the active steroids in the gastrointestinal tract and liver, and the conversion is so fast that, 30 min after ingestion, ethynodiol diacetate cannot be detected in serum. The metabolic pathways of lynoestrenol and ethynodiol diacetate involve ethynodiol as the intermediate. The disposition of the three progestogens is largely similar to that of norethisterone, except that the terminal half-life after ingestion of lynoestrenol is longer (Kuhl, 1990).

(*b*) *Experimental systems*

No data were available to the Working Group.

4.1.11 *Norgestimate*

(*a*) *Humans*

Alton *et al*. (1984) studied the metabolism of ^{14}C-labelled norgestimate in four women over two weeks. An average of 36.8% of the radiolabel was recovered in faeces and 46.8% in urine. Of the urinary metabolites, 57% was released by enzymatic hydrolysis while 12% was unconjugated. The metabolites were separated by chromatography and shown to include norgestrel, 16β-hydroxynorgestrel, 2α-hydroxynorgestrel, 3α,5β-tetrahydronorgestrel, 3,16-dihydroxy-5-tetrahydronorgestrel and an unidentified trihydroxylated metabolite of norgestrel.

McGuire *et al*. (1990) reviewed previous studies on norgestimate and noted that the ^{14}C-labelled compound was rapidly absorbed and reached maximum levels in serum within 0.5–2 h. The estimated half-life for elimination was 45–71 h. The pattern of metabolites separated and identified by gas chromatography and mass spectroscopy indicated the progressive steps of metabolism: norgestimate undergoes hydrolysis at the 17 position, cleavage of the oxime at position 3, followed by reduction of the ketone, hydroxylation in the A and D rings, reduction of the double bond between carbons 4 and 5, and subsequent conjugation to a sulfate or glucuronide. In a study of 10 women who received one or multiple oral doses of 180 μg norgestimate plus 35 μg of ethinyloestradiol, norgestimate was found to be absorbed rapidly, with a maximum serum concentration of 100 pg/mL reached 1 h after treatment. The concentrations declined rapidly thereafter, and none was detectable by 5 h with the techniques used.

The metabolism of norgestimate was investigated in fragments of human colon and in microsomes isolated from human liver. Two hours after addition of labelled norgestimate to the colon tissue, 38% unaltered norgestimate, 49% 17-deacetylnorgestimate and 8.1% conjugated metabolites were found. Five hours after addition of norgestimate to human liver microsomes, there was some deacetylation of norgestimate to 17-deacetylnorgestimate in the absence of NADPH; in the presence of NADPH, only 30% unaltered norgestimate remained, with 39% 17-deacetylnorgestimate, less than 2% 3-ketonorgestimate, 10% norgestrel and 15% unidentified metabolites. The metabolism of 17-deacetylnorgestimate by human liver microsomes was NADPH- and oxygen-dependent and yielded norgestrel and other metabolites (Madden & Back, 1991).

Kuhnz *et al*. (1994b) treated 12 women with single doses of combined oral contraceptives containing either 250 μg levonorgestrel plus 50 μg ethinyloestradiol or 250 μg norgestimate plus 35 μg ethinyloestradiol. About 22% of the dose of norgestimate became available systemically as levonorgestrel.

(*b*) *Experimental systems*

Norgestimate is metabolized mainly to levonorgestrel. In rabbits, norgestimate had no greater androgenic activity than progesterone *in vivo* or *in vitro*. It showed very poor affinity for androgen receptors and did not bind to human sex hormone-binding globulin (Phillips *et al*., 1992).

After subcutaneous administration of norgestimate to immature, castrated male rats and pregnant female rats, levonorgestrel was the principal metabolite. The progestational and androgenic pharmacological responses to treatment with norgestimate were equivalent to those observed at the concentrations of levonorgestrel achieved after that dose (Kuhnz & Beier, 1994).

4.1.12 *Norgestrel*

(*a*) *Humans*

No data were available to the Working Group.

(*b*) *Experimental systems*

Hussain *et al*. (1991) examined the effects of an oral contraceptive containing ethinyloestradiol at 50 μg and norgestrel at 0.5 mg, on hepatic cytochrome P450 and cytochrome b5 activity in microsomes and glutathione *S*-transferase activity in cytosol. Doses spanning two orders of magnitude (1/20th–1/2000th of the pill dose) were administered to mice daily for 15 days before the study *in vitro*. The intermediate doses significantly decreased cytochrome P450 and cytochrome b5 activity and increased the sulfhydryl group concentration but had no effect on glutathione *S*-transferase activity; the highest dose (1/20th of the pill), however, decreased the activity of this enzyme.

4.2 Receptor-mediated effects

4.2.1 *Combined oral contraceptives*

Anderson *et al*. (1989) obtained tissue from breast biopsies taken from 347 pre-menopausal women and determined the incorporation of tritiated thymidine into the DNA of epithelial cells. The labelling index (the percentage of cells that had incorporated tritiated thymidine) was higher in women who used combined oral contraceptives than in women who did not during the first 13 days and last seven days of the menstrual cycle. The difference was significant for days 6–13 (approximately 80% increase for 38–44 women per group) but not for days 21–28 (15–20% increase for 43–49 women per group). Multivariate analysis indicated that the effect of current oral contraceptive use increased cell proliferation significantly ($p < 0.01$); the effect appeared to be confined to nulliparous women ($p < 0.005$). The women reported use of at least 20 different brands of oral contraceptive, and the heterogeneity in response in terms of labelling index was statistically significant in the multivariate analysis. There was an apparent relation between ethinyloestradiol dose and labelling index, which increased from 0.66% (95% CI, 0.52–0.85; $n = 83$) and 0.89% (95% CI, 0.65–1.2; $n = 55$) for users of less than 35 μg per day to 1.3% (95% CI, 0.82–1.9; $n = 15$) for women taking 35 μg per day and 3.5% (95% CI, 3.2–3.9) for two women using 50 μg per day. There was no apparent effect of progestogen dose, which was associated with a labelling index of 0.97–0.98% in 21 women using low-progestogen doses, i.e. norgesterel or desogestrel, and 51 women using high-progestogen doses, i.e. norethisterone, lynoestrenol or ethynodiol acetate. This value was similar to the labelling index found in 36 women using triphasic oral contraceptives (0.94%). In a study by

Williams *et al*. (1991), of similar design, 49 oral contraceptive users were compared with 127 women who were not. The observation of an increased breast epithelial cell labelling index in users during the second week of the menstrual cycle was confirmed. Furthermore, throughout the menstrual cycle, fewer cells expressed oestrogen receptor in users than in non-users, the major difference also occurring during week 2. Two further studies did not, however, find an increased labelling index in breast epithelium of women using combined oral contraceptives (Anderson *et al*., 1982; Going *et al*., 1988). In a smaller study (Olsson *et al*., 1996), breast epithelium staining for Ki-S5 antibody (a marker of DNA synthesis) was investigated in reduction mammoplasty samples from 58 women aged 17–47 years; 18 women were current users of oral contraceptives, 34 were past users, and six had never been exposed. There was no difference in labelling index in the three groups or between parous and nulliparous women. There was, however, a significant increase in labelling in 41 women who had used oral contraceptives before a first full-term pregnancy and in 31 who had used them before the age of 20 in comparison with the other women (n = 17 and 27, respectively). Taken together, these studies clearly demonstrate that combined oral contraceptive use increases breast epithelial cell proliferation; the study of Anderson *et al*. (1989) suggests that the dose of ethinyloestradiol influences the magnitude of this effect in the presence of progestogens.

4.2.2 *Ethinyloestradiol*

(*a*) *Humans*

Odlind *et al*. (1980) studied the effects of combined oral contraceptive use on the concentrations of sex hormone-binding globulin in five healthy pre-menopausal women. A dose of 35 μg per day ethinyloestradiol in combination with 0.5 mg per day norethisterone or a dose of 50 μg per day mestranol combined with 1 mg norethisterone given for the duration of one menstrual cycle increased the concentrations by approximately 100%. Administration of 60 μg ethinyloestradiol every other day in combination with 0.5 mg per day norethisterone caused a 50% increase, and a combination of 50 μg per day ethinyloestradiol with 3 mg norethisterone acetate or 2.5 mg lynoestrenol caused a 20% increase. The same dose of 50 μg per day ethinyloestradiol in combination with 1 mg per day lynoestrenol increased the concentration of sex hormone-binding globulin by approximately 80%.

(*b*) *Experimental systems*

The synthetic oestrogen ethinyloestradiol has been shown to bind to the oestrogen receptor of calf and rabbit uterus, rat liver and human oviduct (Kappus *et al*., 1973; Eisenfeld *et al*., 1978; Muechler & Kohler, 1980; Powell-Jones *et al*., 1980; Aten & Eisenfeld, 1982; Lubahn *et al*., 1985). Its relative binding affinity to the human oviductal receptor was about equal to that of oestradiol (Muechler & Kohler, 1980). Binding to the calf uterine receptor was 2–2.5 times higher than that of oestradiol (Lubahn *et al*., 1985).

Ethinyloestradiol transiently enhanced replicative DNA synthesis (tritiated thymidine incorporation) in female rat liver. After subcutaneous implantation of time-release pellets

providing 2.5 μg/rat ethinyloestradiol per day, DNA synthesis peaked between 24 and 72 h and slowly returned to control values within 7–14 days (Yager *et al*., 1986). Similar findings were obtained with doses of 2 μg/kg per day to 3 mg/kg per day delivered by subcutaneous injection. A daily dose of 0.5 mg/kg (approximately 80 μg/rat) caused an increase in liver weight (by about 60% in comparison with pair-fed controls, the latter showing a 35% reduction in liver weight in comparison with control rats fed *ad libitum*) and in liver DNA content (by approximately 30% in comparison with pair-fed controls) (Ochs *et al*., 1986). For these two effects, the relationship between dose and response was approximately log-linear over the range of doses tested (Ochs *et al*., 1986; Schulte-Hermann *et al*., 1988). Oral administration of ethinyloestradiol was less effective than subcutaneous injection (Ochs *et al*., 1986). The effects at the lower doses are probably mediated by the oestrogen receptor, because the increase in DNA synthesis was inhibited by treatment with the anti-oestrogen tamoxifen (15 μg/rat per day), which by itself did not alter hepatic DNA synthesis (Yager *et al*., 1986).

Prolonged exposure of female rats to ethinyloestradiol at a dose of 2.5 or 5 μg/rat per day from time-release pellets stimulated replicative DNA synthesis in the liver during the first week, but strongly inhibited this process after 28 days (72% inhibition) and 42 days (88% inhibition) of ethinyloestradiol treatment in comparison with untreated controls. Treatment with 5 μg/rat per day ethinyloestradiol for 21 days inhibited the regenerative growth response (tritiated thymidine incorporation) usually seen during the first four days after partial hepatectomy. Epidermal growth factor receptor levels were decreased after seven days of ethinyloestradiol treatment, but had returned to control levels after 21 days (Yager *et al*., 1994).

Moser *et al*. (1996) treated 12-day-old B6C3F1 mice with a single intraperitoneal dose of 5 mg/kg bw NDEA followed four weeks later by administration of ethinyloestradiol at 1 mg/kg diet for 16 weeks. Treatment with NDEA and oestrogen did not change the DNA labelling index (0.4–0.8%; bromodeoxyuridine (BrdU) incorporation) observed in normal hepatocytes, but ethinyloestradiol reduced by approximately 70% the markedly increased labelling index (18%) caused by NDEA in hepatic foci of cellular alteration. This ethinyloestradiol-induced decrease in DNA synthesis in foci was accompanied by a decrease in the size of these foci and by a reduction in the number of foci with a decreased (as compared with normal hepatocytes) content of transforming growth factor (TGF)-β1 and of the mannose-6-phosphatase/insulin–like growth factor-II receptor, which is involved in activation of latent TGF-β1.

Vickers *et al*. (1989) and Vickers and Lucier (1991, 1996) gave ovariectomized rats a single intraperitoneal dose of 200 mg/kg bw NDEA, followed by a daily dose of 90 μg/kg bw ethinyloestradiol by slow-release implant for 30 weeks. This treatment restored the decreased liver weights to the values in intact controls, increased the uterine weights above those of intact controls, and restored the decreased total nuclear and cytosolic oestrogen receptor concentrations and the nuclear hepatic oestrogen receptor occupancy in elutriated hepatic parenchymal cells to values greater than those in intact controls. Pretreatment with the chemical carcinogen slightly enhanced these effects of

ethinyloestradiol (Vickers & Lucier, 1991). Very similar effects were found in isolated hepatic sinusoidal endothelial and Kupffer cells enriched by centrifugal elutriation. These cell fractions, derived from female rats treated with a single intraperitoneal dose of 200 mg/kg bw NDEA with or without ethinyloestradiol at 90 µg/kg bw per day for 30 weeks, showed a 5–6.5-fold increase in nuclear oestrogen receptor levels and a two- to three-fold increase in receptor occupancy (Vickers & Lucier, 1996).

In vitro, ethinyloestradiol at 15×10^{-6} mol/L induced mitogenesis in primary cultures of female rat hepatocytes, increasing tritiated thymidine incorporation by two- to three-fold 30 h after exposure (Shi & Yager, 1989; Ni & Yager, 1994a). Although ethinyloestradiol by itself therefore appeared to have only weak mitogenic effects on rat hepatocytes, as a co-mutagen with epidermal growth factor it strongly enhanced the induction of hepatic DNA synthesis when this factor was added during the last 12 h of the 30-h exposure to oestrogen. Thus, Shi and Yager (1989) demonstrated that 25 ng/mL epidermal growth factor increased tritiated thymidine incorporation by almost ninefold at an ethinyloestradiol concentration of 2.5 µmol/L, and almost 14-fold at 15 µmol/L. An 18-h exposure to 2 µmol/L ethinyloestradiol doubled the number of epidermal growth factor receptors per cell, providing a rational explanation for the increased sensitivity of ethinyloestradiol-exposed hepatocytes to epidermal growth factor. A similar effect occurred *in vivo* 24 h after a single 2.5-µg dose of ethinyloestradiol given to female rats: binding of radiolabelled epidermal growth factor started to increase after 8 h and reached twofold maximum enhancement after 18 h. The amount of epidermal growth factor receptor protein increased proportionally, and its half-life increased by 4.3-fold, while receptor mRNA synthesis was not affected by ethinyloestradiol. Thus, stabilization of the epidermal growth factor receptor protein appeared to be the mechanism by which ethinyloestradiol co-stimulated epidermal growth factor-induced mitogenesis in female rat hepatocytes. Epidermal growth factor-induced growth of male rat hepatocytes, however, was inhibited by oestrogen treatment (Francavilla *et al.*, 1989). Although this result suggests that marked sex differences exist in the mitogenic effects of epidermal growth factor and oestrogens on rat liver, the use of a different cell culture medium may also have played a role (Yager & Liehr, 1996).

In the presence of 30 nmol/L dexamethasone, ethinyloestradiol treatment at concentrations of 1×10^{-5}–3×10^{-5} mol/L for five days induced γ-GT activity in cultured rat hepatocytes (Edwards & Lucas, 1985).

4.2.3 *Mestranol*

(*a*) *Humans*

Odlind *et al*. (1980) studied the effects of combined oral contraceptive use on the plasma concentrations of sex hormone-binding globulin in five healthy pre-menopausal women. A dose of 50 µg/day mestranol in combination with 1.0 mg/day norethisterone given for the duration of one menstrual cycle to five pre-menopausal women increased the concentration of sex hormone-binding globulin by approximately 100%.

(*b*) *Experimental systems*

Mestranol does not bind to the oestrogen receptor in rabbit uterus (Kappus *et al*., 1973) but bound to those in calf uterus and human oviduct (Muechler & Kohler, 1980; Lubahn *et al*., 1985), although its relative binding affinity was about two orders of magnitude less than that of oestradiol or ethinyloestradiol.

Mestranol caused a threefold increase in replicative DNA synthesis in female rat liver 24 h after the insertion of slow-release pellets delivering 2.5 or 5 μg/rat per day. This effect may be mediated by the oestrogen receptor because the increase in DNA synthesis induced by 2.5 μg per day mestranol was inhibited by concomitant treatment with 15 μg/rat per day tamoxifen, which by itself did not alter hepatic DNA synthesis (Yager *et al*., 1986). The mestranol-induced increase in DNA synthesis was confirmed in experiments in which mestranol was given at a dose of 0.2 mg/kg diet to female rats for eight months. This treatment effectively promoted the induction of enzyme-altered foci in the liver by a single dose of NDEA, as judged from a threefold increase in the BdrU incorporation index in these preneoplastic foci relative to the labelling index in the surrounding normal hepatocytes, which was also increased (Dragan *et al*., 1996).

In vitro, mestranol at doses of 10^{-8}–10^{-5} mol/L enhanced mitogenesis in HepG2 human hepatocarcinoma cells by up to 80% in comparison with control cells (Coezy *et al*., 1987), and it was co-mitogenic at 10^{-6}–10^{-5} mol/L in primary female rat hepatocytes cultured in the presence of TGF-α (Ni & Yager, 1994b). In another study, however, mestranol inhibited the growth of Hep3B human hepatoma cells at 10^{-5} mol/L under conditions in which it did not significantly affect the growth of HepG2 cells (Jiang *et al*., 1995). Tamoxifen at 10^{-6}–10^{-5} mol/L eliminated the mestranol-induced mitogenesis in oestrogen receptor-containing HepG2 carcinoma cells, which points to an oestrogen-mediated mechanism (Coezy *et al*., 1987). In Hep3B hepatoma cells, which do not express oestrogen receptor, tamoxifen inhibits all cell growth. In the presence of mestranol, an additive inhibitory effect was observed, which suggests that the growth inhibition by mestranol observed in these cells is an oestrogen receptor-independent process (Jiang *et al*., 1995).

Mestranol *per se* induced γ-GT activity in cultured rat hepatocytes at concentrations of 3×10^{-6}–10^{-4} mol/L in the presence of 30 nmol/L dexamethasone (Edwards & Lucas, 1985).

The combination of norethynodrel (0.5 or 5 mg/rat per day) and mestranol (7.5 or 75 μg/rat per day) given as a pellet implant to female Sprague-Dawley rats, starting at 45, 55, 65 or 75 days of age, caused changes in the mammary gland that resulted in protection against induction of mammary cancer by a single dose (80 mg/kg bw) of 7,12-dimethylbenz[*a*]anthracene (DMBA) (Russo *et al*., 1989). The hormone treatment was given for 21 days, followed by 21 days' recovery, at which time some rats were killed to study mammary gland morphology, while others received DMBA. The hormone treatment at both doses decreased the number of terminal end-buds per mammary gland and increased the number of alveolar buds, but did not alter the number of terminal ducts; cell proliferation, measured as the DNA-labelling index, was reduced in the terminal ducts and alveolar buds but remained unchanged in the terminal end-buds (Russo *et al*.,

1989; Russo & Russo, 1991). In these experiments, a trend was observed for the hormonal treatment to produce less effect when initiated at a later age. The reduction in cell proliferation in terminal end-buds and terminal ducts, the target tissues for DMBA, may explain the protective effect of the hormone combination on the development of mammary cancer.

In a study with rhesus monkeys (Tavassoli *et al*., 1988), mestranol alone at 0.02 or 0.1 mg/kg per day and combinations of mestranol and ethynerone, chlorethinyl norgestrel and anagestone acetate were given for 10 years in 28-day cycles consisting of 21 days of administration followed by seven days without treatment. Mestranol alone induced minimal to moderate proliferative and atypical alterations in the mammary gland in 8/34 animals, whereas minimal to mild changes occurred in 2/16 controls. With the various mestranol–progestogen combinations, mild to severe atypical hyperplasia was observed in 22–25/52 animals, about 12% in each group showing severe lesions that could not be distinguished from human mammary carcinoma *in situ*. Minimal to severe proliferative atypia were found in 11/15 animals given one of the progestogens, ethynerone; two of these animals had a severe lesion similar to carcinoma *in situ* and one had invasive breast cancer.

4.2.4 *Chlormadinone acetate*

(*a*) *Humans*

No relevant data were available to the Working Group.

(*b*) *Experimental systems*

The progestogen chlormadinone acetate inhibited the induction by ethinyloestradiol of nuclear and cytoplasmic progesterone receptor in human endometrium (Kreitmann *et al*., 1979), and it has been found to bind strongly to the human uterine progesterone receptor, as determined in a competitive binding assay with the 20 000 × *g* supernatant fraction of human endometrium and myometrium (Briggs, 1975). It reduced the binding of oestradiol to rat uterine oestrogen receptor both *in vivo* and *in vitro* (Di Carlo *et al*., 1983). Chlormadinone acetate did not have any detectable oestrogenic activity when tested for induction of alkaline phosphatase activity as an indicator of oestrogen response in oestrogen receptor-containing and oestrogen-sensitive Ishikawa human endometrial cancer cells (Botella *et al*., 1995).

In vitro, chlormadinone acetate at 10^{-6} mol/L stimulated the growth of androgen-sensitive mouse mammary carcinoma Shionogi cells, with a reduction in doubling time of approximately 50%. This effect could be inhibited by a 5×10^{-6} mol/L excess of the androgen receptor-blocking anti-androgen hydroxyflutamide, which by itself did not stimulate the growth of these cells (Luthy *et al*., 1988). Consistent with these observations, chlormadinone acetate weakly bound to the rat ventral prostate androgen receptor (Botella *et al*., 1987).

The growth stimulatory effect of chlormadinone acetate on Shionogi cells was confirmed *in vivo* in DD/S mice: the tumour size was increased by more than threefold

over that in controls after 21 days of treatment with two daily dose of 250 μg/mouse (Plante *et al.*, 1988). When tested in castrated male rats at a dose of 10 mg twice daily for 14 days, chlormadinone acetate increased ventral prostate weight by about 50% and stimulated the activity of the cell proliferation-related enzyme ornithine decarboxylase in the ventral prostate by almost 12-fold; effects of similar magnitude were found with 5α-dihydrotestosterone at a dose of 0.15 mg twice daily. Thus, chlormadinone acetate has weak androgenic activity, while no evidence for anti-androgenic activity was detected in these studies (Labrie *et al.*, 1987).

Studies with the human breast cancer cell line ZR-75-1, which contains functional oestrogen, progesterone and androgen receptors, suggested that chlormadinone acetate inhibited the growth of these cells by an interaction of androgen and progesterone receptor-mediated mechanisms (Poulin *et al.*, 1990).

Chlormadinone acetate inhibited the activity of microsomal oestrone sulfatase in human breast carcinoma tissue *in vitro*, suggesting that it may reduce the formation of biologically active oestrogen in human breast cancer cells *in vivo* (Prost-Avallet *et al.*, 1991). It also reduced the activity of 5α-reductase and increased the activity of hepatic 3α- and 3β-hydroxysteroid dehydrogenase in male and female rats (Lax *et al.*, 1984).

4.2.5 *Cyproterone acetate*

(*a*) *Humans*

No relevant data were available to the Working Group.

(*b*) *Experimental systems*

Cyproterone acetate is an anti-androgen that has been shown to act at the level of both the (peripheral) androgen receptor and the hypothalamus-pituitary, suppressing gonadotrophin release. Interestingly, it also had intrinsic androgenic activity when tested for its ability to increase the weight of the ventral prostate of castrated male rats (Poyet & Labrie, 1985). In comparison with 5α-dihydrotestosterone, however, it bound only weakly to the rat ventral prostate androgen receptor (Botella *et al.*, 1987). In a test system comprising steroid receptor-deficient CV-1 monkey kidney cells stably transfected with androgen receptor and a reporter plasmid containing the mouse mammary tumour virus promotor linked to the chloramphenicol acetyltransferase gene, transcriptional activation of chloramphenicol acetyltransferase has been used to show both androgenic activity of cyproterone acetate (Warriar *et al.*, 1993) and lack of androgenic activity (Fuhrmann *et al.*, 1992). The human androgen receptor was used in the former study and the rat androgen receptor in the latter, but it is not clear whether this difference was responsible for the discordant findings. In both studies, excess cyproterone acetate inhibited the effect of androgens.

Cyproterone acetate stimulated the growth of androgen-sensitive mouse mammary carcinoma Shionogi cells *in vivo* in DD/S mice; the tumour size was increased 11-fold over that in controls after 21 days of treatment with two daily doses of 250 μg/mouse (Plante *et al.*, 1988).

Cyproterone acetate did not stimulate and, indeed, even inhibited the growth of the original MCF-7 human breast cancer cell line at concentrations of 10^{-7}–10^{-5} mol/L, as measured by tritiated thymidine incorporation (Lippman *et al.*, 1976). In a later study, stimulation of the growth of the oestrogen-sensitive breast cancer cell lines MCF-7 and EFM-19 was found at concentrations of 10^{-8}–10^{-6} mol/L cyproterone acetate. This effect was influenced by competition with 5α-dihydrotestosterone but not oestradiol, indicating involvement of the androgen receptor but not the oestrogen receptor (Hackenberg *et al.*, 1988). In contrast, studies with the human breast cancer cell line ZR-75-1, which contains functional oestrogen, progesterone and androgen receptors, indicated that cyproterone acetate inhibits the growth of these cells, suggesting that this occurs via an interaction of androgen and progesterone receptor-mediated mechanisms (Poulin *et al.*, 1990).

Cyproterone acetate is also a progestogen and has been demonstrated to bind to the progesterone receptor of human uterus (Grill *et al.*, 1985) and MCF-7 human breast cancer cells (Bergink *et al.*, 1983). Cyproterone acetate had oestrogenic activity in ovariectomized mice, as was evident from the observed vaginal keratinization and increases in uterine weight and protein content (Lohiya & Arya, 1981). It did not alter the uterine hyperplastic response to conjugated equine oestrogen in ovariectomized rats (Kumasaka *et al.*, 1994).

Cyproterone acetate has considerable effects on the rodent liver: it stimulates the proliferation of hepatocytes, resulting in liver enlargement due to hyperplasia, in the absence of hepatotoxic effects. After three to six daily administrations by gavage of 40–130 mg/kg cyproterone acetate dissolved in oil to female and male rats, the increase in the ratio of liver weight:body weight reached a plateau at 1.5 times to more than twice the values in vehicle-treated controls, while the hepatic DNA content nearly doubled (Bursch *et al.*, 1986; Schulte-Hermann *et al.*, 1988; Roberts *et al.*, 1995). A threshold dose of 5–10 mg/kg per day was found for these effects in female Wistar rats, male rats being less sensitive and showing less pronounced growth of the liver. With a lag of 12–14 h, replicative DNA synthesis was induced by cyproterone acetate in female Wistar rats, reaching a maximum 18–24 h after the first dose, with a predominant response of periportal hepatocytes (Schulte-Hermann *et al.*, 1980a). Cyproterone acetate given at a dose of 125 mg/kg bw per day in the diet to C57BL/10J mice increased the BrdU nuclear labelling index in the liver, the effect being statistically significant in females (Tucker & Jones, 1996; Tucker *et al.*, 1996). As many as 75% of all hepatocytes responded to cyproterone acetate with proliferation (Schulte-Hermann *et al.*, 1980b). Several studies have demonstrated that after cessation of cyproterone acetate treatment, the liver regresses to its normal size, due to massive induction of apoptosis (Bursch *et al.*, 1986; Roberts *et al.*, 1995). Cyproterone acetate induced the synthesis of TGF-β1 which is possibly involved in the apoptotic response of hepatocytes after withdrawal of cyproterone acetate (Bursch *et al.*, 1993; Oberhammer *et al.*, 1996).

The mitogenic activity of cyproterone acetate in rat hepatocytes is apparently a direct effect, since the compound stimulated replicative DNA synthesis in female rat hepatocytes cultured in serum-free medium at non-cytotoxic concentrations of 10^{-7}–10^{-4} mol/L

(Parzefall *et al*., 1989); however, proliferation of hepatocytes isolated from human surgical specimens was, on average, not increased by exposure to cyproterone acetate for 24 h at concentrations of 10^{-5} mol/L. This lack of effect was seen with and without subsequent addition of epidermal growth factor during 24 h. In contrast, cyproterone acetate and epidermal growth factor acted in an additive manner in stimulating DNA synthesis in rat hepatocytes, whereas epidermal growth factor *per se* enhanced the growth of both human and rat cultured liver cells (Parzefall *et al*., 1991). The observations with human hepatocytes were limited to cells obtained from seven subjects; while in most cases no effect was observed, a dose-related increase in proliferation was induced by cyproterone acetate in hepatocytes from one of the subjects and a dose-related decrease in cells from another. More observations are therefore needed before a firm conclusion can be reached about the possible proliferative effects of cyproterone acetate on human liver.

Cyproterone acetate caused a shift of the cell cycle of cultured rat hepatocytes from G_0 to the G_1 phase (Duivenvoorden & Maier, 1994), with concomitant induction of c-*myc* and c-*fos* expression (Duivenvoorden *et al*., 1995).

Female rats were subjected at six weeks of age to a carcinogenic regimen of a two-thirds hepatectomy followed 20 h later by gastric intubation with 30 mg/kg NDEA and, one week later, administration of 0.1% phenobarbital in the drinking-water for four to six months. In cultured hepatocytes derived from three rats, cyproterone acetate at 5×10^{-6} mol/L induced a fourfold increase in replicative DNA synthesis in putatively preneoplastic γ-GT-positive cells and a twofold increase in γ-GT-negative hepatocytes. These effects required the presence of both epidermal growth factor and insulin, which by themselves increased proliferation 10-fold over that in controls but did not differentially affect the proliferation of γ-GT-positive and γ-GT-negative cells (Neumann *et al*., 1992). In the same series of experiments, stimulation of DNA repair synthesis by cyproterone acetate was observed in hepatocytes from both carcinogen-treated and untreated rats, and in medium without epidermal growth factor. This raises the possibility that cyproterone acetate has tumour-initiating potential. Cyproterone acetate *per se* at concentrations of 10^{-6}–10^{-5} mol/L induced γ-GT activity in cultured rat hepatocytes (Edwards & Lucas, 1985).

The exact mechanism by which cyproterone acetate induces liver-cell proliferation and hepatic hyperplasia is not understood. Although it stimulated incorporation of tritiated thymidine into cultured hepatocytes from carcinogen-treated female rats at concentrations of 2×10^{-6}–10^{-5} mol/L, another anti-androgen, flutamide, inhibited stimulation of hepatocyte proliferation induced by epidermal growth factor and insulin (Neumann *et al*., 1992). These findings suggest that the hyperplastic effects of cyproterone acetate are not related to its anti-androgenic properties. It is, however, conceivable that the effects are, at least in part, related to the aforementioned androgenic properties of cyproterone acetate, possibly mediated by the androgen receptor. Unlike compensatory liver cell proliferation, which occurs in rats in response to surgical or toxic reduction of the liver mass, direct hepatic hyperplasia induced by cyproterone acetate *in vivo* did not involve up-regulation of the immediate–early response proto-oncogenes c-*fos*, c-*jun* and c-*myc* or induction of

the transcription factors NF-κB and AP-1 (Coni *et al*., 1993; Menegazzi *et al*., 1997). *In vitro*, however, cyproterone acetate-induced hepatocyte proliferation was accompanied by increased expression of not only c-*fos* but also c-*myc* (Duivenvoorden *et al*., 1995). These observations suggest that the stimulation of rat hepatocyte proliferation by cyproterone acetate *in vivo* may differ from that in culture.

Cyproterone acetate also increased the activity of 5α-reductase and decreased the activity of 3α- and 3β-hydroxysteroid dehydrogenases in the livers of male and female rats (Lax *et al*., 1984).

4.2.6 *Desogestrel*

(*a*) *Humans*

Ruokonen and Käär (1985) studied the effects of desogestrel at a dose of 125 μg per day for 60 days in 30 healthy pre-menopausal women with regard to the serum levels of ceruloplasmin and cortisol-binding globulin, as indicators of oestrogenic activity. The concentrations of these proteins were not affected by the treatment, indicating a lack of oestrogenic activity of desogestrel in these women. The serum concentration of sex hormone-binding globulin was markedly decreased by the treatment, to 70 and 60% of pre- and post-treatment values at 30 and 60 days, respectively.

(*b*) *Experimental systems*

The progestogen desogestrel is converted to its unique, directly acting metabolite 3-keto-desogestrel, and this metabolite was used in all of the studies conducted *in vitro*. The relative binding affinity of 3-keto-desogestrel to the rabbit uterine progesterone receptor has been reported to be approximately equal to (Fuhrmann *et al*., 1995) or nine times higher than that of progesterone (Phillips *et al*., 1990).

The progestational activity of desogestrel *in vivo*, measured by inhibition of ovulation and endometrial stimulation in rabbits, was similar to that of progesterone (Phillips *et al*., 1987).

The 3-keto metabolite of desogestrel also bound with high affinity to androgen and glucocorticoid receptors (Kloosterboer *et al*., 1988; Juchem & Pollow, 1990; Phillips *et al*., 1990; Fuhrmann *et al*., 1995), but not to oestrogen or mineralocorticoid receptors (Juchem & Pollow, 1990; Fuhrmann *et al*., 1995; Schoonen *et al*., 1995a,b). The relative binding affinity for the rat ventral prostate androgen receptor was approximately 12% that of dihydrotestosterone (Phillips *et al*., 1990). In *trans*-activation assays, 3-keto-desogestrel had clear androgenic activity and weak glucocorticoid activity, but no agonist or antagonist activity was found in assays that involved the mineralocorticoid receptor (Fuhrmann *et al*., 1995).

In comparison with 5α-dihydrotestosterone, desogestrel had modest androgenic activity *in vivo*, as measured by stimulation of ventral prostate growth in castrated rats (Phillips *et al*., 1987).

3-Keto-desogestrel stimulated the growth of most oestrogen-sensitive human mammary cancer cell lines tested (van der Burg *et al*., 1992; Kalkhoven *et al*., 1994;

Schoonen *et al.*, 1995a,b). MCF-7 cell proliferation was stimulated by 3-keto-desogestrel at a concentration of 10^{-6} mol/L, but only in the presence of insulin added to the medium at ≥ 10 ng/mL (van der Burg *et al.*, 1992). In experiments in which growth stimulation by 3-keto-desogestrel was compared in various cell lines, it did not appear to require insulin or epidermal growth factor. The growth stimulation was dose-dependent, beginning at concentrations of 10^{-7} mol/L for MCF-7 cells and 10^{-10} mol/L for T47D cells obtained from two different sources (Kalkhoven *et al.*, 1994). These dose–response results were confirmed in studies in which the same and two additional sub-lines of MCF-7 and one of the T47D cell lines were used, while the other T47D line did not respond to 3-keto-desogestrel (Schoonen *et al.*, 1995a,b). The experiments were performed with breast cancer cell lines grown in phenol red-free medium containing steroid-devoid (dextran-coated charcoal-stripped) serum (van der Burg *et al.*, 1992; Kalkhoven *et al.*, 1994; Schoonen *et al.*, 1995a,b). Under the conditions of these experiments, progesterone receptor expression in both MCF-7 and T47D cells was maintained and was 40-fold higher in the T47D than in the MCF-7 cells. Furthermore, expression of the progesterone-inducible gene encoding fatty acid synthase was more strongly up-regulated by 3-keto-desogestrel in T47D than in MCF-7 cells. By use of a reporter construct containing two progesterone response elements in front of the thymidine kinase promotor coupled to the chloramphenicol acetyltransferase gene transfected into both cell lines, progesterone receptor-mediated *trans*-activation was observed at a concentration of 3-keto-desogestrel as low as 10^{-9} mol/L (Kalkhoven *et al.*, 1994). In other experiments, the stimulating effects on cell growth of 3-keto-desogestrel at concentrations of 10^{-7}–10^{-6} mol/L were not blocked by simultaneous treatment of the cells with anti-progestogens such as RU486, whereas the anti-oestrogens 4-hydroxytamoxifen and ICI164,384 (at 10^{-7} mol/L) did inhibit this stimulation (van der Burg *et al.*, 1992; Schoonen *et al.*, 1995a,b). Growth stimulation of T47D cells by 3-keto-desogestrel at 10^{-10} mol/L was inhibited by RU486 and not by 4-hydroxytamoxifen (both at 10^{-7} mol/L) (Kalkhoven *et al.*, 1994). These findings suggest that stimulation of cell proliferation by 3-keto-desogestrel is mediated by the oestrogen receptor at high concentrations and by the progesterone receptor at low concentrations. This is apparently not related to effects at the level of receptor–ligand interaction: 3-keto-desogestrel causes *trans*-activation of reporter constructs containing oestrogen or progesterone response elements transfected into MCF-7 and T47D cells at 10^{-6} and 10^{-9} mol/L, respectively, while 4-hydroxytamoxifen, but not RU486, inhibited *trans*-activation of the oestrogen response element-containing construct, and RU486, but not 4-hydroxytamoxifen, inhibited *trans*-activation of the progesterone response element-containing construct. The expression of the oestrogen-inducible *pS2* gene in MCF-7 cells was slightly inhibited by 3-keto-desogestrel at 10^{-9} mol/L and was not affected at 10^{-6} mol/L (Kalkhoven *et al.*, 1994).

Oestradiol at concentrations of 10^{-10} mol/L and higher strongly induced the growth of the MCF-7 and T47D cell lines, regardless of the sub-line used (van der Burg *et al.*, 1992; Kalkhoven *et al.*, 1994; Schoonen *et al.*, 1995a,b). The growth stimulation of MCF-7 cells by oestrogen at 10^{-10} mol/L was inhibited by 3-keto-desogestrel at a concentration of

10^{-8} mol/L but not by the anti-progestogen RU38486 (Schoonen *et al.*, 1995a). Oestrogen-induced growth in T47D cells was not blocked by 3-keto-desogestrel at 10^{-6} mol/L in one sub-line (T47D-A) but was totally inhibited in another sub-line (T47D-S) at a concentration of 10^{-10}–10^{-8} mol/L. These two sub-lines differ considerably, in that RU38486, but not 4-hydroxytamoxifen or ICI164,384, blocked oestrogen-stimulated growth in the T47D-A cell line, while both anti-progestogens and anti-oestrogens inhibited T47D-S (Schoonen *et al.*, 1995b).

3-Keto-desogestrel at concentrations of 10–40 ng/mL inhibited the growth of endothelial cells derived from human decidual endometrium; the growth of these cells was stimulated by exposure to oestradiol at 5 ng/mL (Peek *et al.*, 1995).

3-Keto-desogestrel, but not the parent compound desogestrel, showed moderate affinity for and slow dissociation from sex hormone-binding globulin in human serum (Juchem & Pollow, 1990). Its strong interaction with this globulin could lead to displacement of testosterone and to an increased concentration of free testosterone; however, the decrease in serum sex hormone-binding globulin after progestogen treatment is probably more important in this respect (Nilsson & von Schoultz, 1989).

4.2.7 *Ethynodiol diacetate*

(*a*) *Humans*

No relevant data were available to the Working Group.

(*b*) *Experimental systems*

The progestogen, ethynodiol diacetate, binds with low affinity (K_i 1.3×10^{-7} mol/L) to both the oestrogen and the progesterone receptor in rabbit uterine cytosol (Tamaya *et al.*, 1977) but hardly at all to the human endometrial progesterone receptor (Briggs, 1975; Shapiro *et al.*, 1978). It also has been reported to have androgenic properties (Darney, 1995), but no information was available on its receptor-mediated effects.

4.2.8 *Gestodene*

(*a*) *Humans*

No relevant data were available to the Working Group.

(*b*) *Experimental systems*

The progestogen gestodene binds to the rabbit uterine progesterone receptor with a relative binding affinity reported to be similar (Fuhrmann *et al.*, 1995) or nine times higher than that of progesterone itself (Phillips *et al.*, 1990), and 8–10 times higher than that of progesterone in human endometrial, breast and liver tissue (Iqbal & Colletta, 1987).

Gestodene also bound with high to moderate affinity to the androgen, mineralocorticoid and glucocorticoid receptors (Kloosterboer *et al.*, 1988; Juchem & Pollow, 1990; Phillips *et al.*, 1990; Fuhrmann *et al.*, 1995), but did not bind to the oestrogen receptor (Juchem & Pollow, 1990; Pollow *et al.*, 1990; Fuhrmann *et al.*, 1995). Oestrogen receptor

binding of gestodene has, however, been reported to occur in malignant breast tissue with threefold higher affinity than that of oestradiol (Iqbal *et al*., 1986). Oestradiol and tamoxifen did not interfere with gestodene binding, but gestodene in excess amounts could reduce oestradiol binding (Iqbal & Valyani, 1988). High-affinity binding of gestodene was found in all breast cancer cell lines tested, but not in endometrial carcinoma cells. Cytosolic gestodene binding could not be inhibited by excess oestradiol, although nuclear binding was abolished (Colletta *et al*., 1989). On the basis of these observations, a novel binding site was postulated (Iqbal & Valyani, 1988; Colletta *et al*., 1989). These findings should be re-evaluated in the light of the identification of the oestrogen receptor-β and current knowledge about oestrogen receptor action. The relative binding affinity of gestodene for the rat ventral prostate androgen receptor was approximately 15% that of dihydrotestosterone (Phillips *et al*., 1990). In *trans*-activation assays, gestodene had clear androgenic activity and weak glucocorticoid activity, but antagonist activity was found for the mineralocorticoid receptor (Fuhrmann *et al*., 1995).

Gestodene has been shown to be a potent competitor for binding of 5α-dihydrotestosterone to the androgen receptor in human foreskin fibroblasts, with activity similar to that of testosterone (Breiner *et al*., 1986).

Gestodene stimulated the growth of most oestrogen-sensitive human mammary cancer cells lines tested (van der Burg *et al*., 1992; Catherino *et al*., 1993; Kalkhoven *et al*., 1994; Schoonen *et al*., 1995a,b). In one study, stimulation of cell proliferation by gestodene at a concentration of 10^{-6} mol/L was found in MCF-7 cells but only in the presence of insulin at ≥ 10 ng/mL (van der Burg *et al*., 1992). In subsequent experiments, stimulation by gestodene was compared in various cell lines and appeared not to require insulin or epidermal growth factor. Furthermore, a dose-dependent stimulation of cell growth was observed, beginning at a concentration of 10^{-7} mol/L for MCF-7 cells and 10^{-10} mol/L for T47D cells obtained from two sources (Kalkhoven *et al*., 1994). In other experiments with similar but not identical culture conditions, gestodene induced near-maximal growth stimulation of MCF-7 cells, at a concentration of 10^{-7} mol/L (Catherino *et al*., 1993). These dose–response results were confirmed in studies with the same and two additional sub-lines of MCF-7; one of two T47D sub-lines tested did not respond to gestodene (Schoonen *et al*., 1995a,b). All of the experiments were performed with breast cancer cell lines grown in phenol red-free medium which, except in one study (Catherino *et al*., 1993), contained steroid-free (dextran-coated charcoal-stripped) serum (van der Burg *et al*., 1992; Kalkhoven *et al*., 1994; Schoonen *et al*., 1995a,b). Under the conditions of these experiments, progesterone receptor expression in both cell types was maintained and was 20–40-fold higher in the T47D cells than in the MCF-7 cells (Kalkhoven *et al*., 1994; see also Sutherland *et al*., 1988). Furthermore, expression of the progesterone-inducible gene encoding fatty acid synthase was more strongly up-regulated by gestodene in T47D than in MCF-7 cells (Kalkhoven *et al*., 1994). With reporter constructs containing two progesterone response elements in front of the *tk* promotor coupled to the chloramphenicol acetyltransferase gene transfected into both cell lines, *trans*-activation was observed at gestodene concentrations as low as 10^{-9} mol/L, clearly demonstrating expression of functional

progesterone receptor in these cell lines without (Kalkhoven *et al*., 1994) or with addition of oestradiol to the medium to boost receptor expression (Catherino *et al*., 1993). The stimulating effects of gestodene at 10^{-7}–10^{-6} mol/L were not blocked, however, by simultaneous treatment of the cells with anti-progestogens such as RU38486, whereas they were inhibited by the anti-oestrogens 4-hydroxytamoxifen (10^{-7} mol/L) and ICI164,384 (10^{-7}–10^{-6} mol/L) (van der Burg *et al*., 1992; Catherino *et al*., 1993; Schoonen *et al*., 1995a,b). Stimulation of the growth of T47D cells by gestodene at a lower concentration (10^{-10} mol/L) was inhibited by RU486 and not by 4-hydroxytamoxifen (both at 10^{-7} mol/L), suggesting that the cell proliferation-stimulating effects of gestodene are mediated via the oestrogen receptor at high concentrations and by the progesterone receptor at low concentrations (Kalkhoven *et al*., 1994). This effect is apparently not related to effects at the level of receptor–ligand interaction, because gestodene causes *trans*-activation of reporter constructs containing oestrogen or progesterone response elements transfected into MCF-7 and T47D cells at 10^{-10}–10^{-6} mol/L. Furthermore, 4-hydroxytamoxifen and ICI164,384, but not RU486, inhibited *trans*-activation of the oestrogen response element-containing construct, while RU486, but not 4-hydroxytamoxifen or ICI164,384, inhibited *trans*-activation of the progesterone response element-containing construct (Catherino *et al*., 1993; Kalkhoven *et al*., 1994). The expression of the oestrogen-inducible *pS2* gene in MCF-7 cells was slightly inhibited by gestodene at a low concentration (10^{-9} mol/L), but was not affected at 10^{-6} mol/L (Kalkhoven *et al*., 1994).

Oestradiol at concentrations of 10^{-10} mol/L and higher strongly induces the growth of the MCF-7 and T47D cell lines, regardless of the sub-line used (van der Burg *et al*., 1992; Kalkhoven *et al*., 1994; Schoonen *et al*., 1995a,b). The growth stimulation of MCF-7 cells by oestrogen at 10^{-10} mol/L was inhibited by gestodene at a concentration of 10^{-8} mol/L, and this effect was not blocked by RU38486 (Schoonen *et al*., 1995a). Oestrogen-induced growth in T47D cells was not blocked by gestodene at 10^{-6} mol/L in one sub-line (T47D-A) but was totally inhibited in another sub-line (T47D-S) at a concentration of 10^{-10}–10^{-8} mol/L. These two sub-lines differ considerably, in that RU38486, but not 4-hydroxytamoxifen or ICI164,384, blocked oestrogen-stimulated growth in the T47D-A cell line, while both anti-progestogens and anti-oestrogens were inhibitory for T47D-S (Schoonen *et al*., 1995b).

Gestodene induced a large increase in secretion of TGF-β by T47D breast cancer cells, but not HEC-1B human endometrial cancer cells, and the inhibitory effect of gestodene on oestrogen-stimulated T47D cell proliferation was reduced by treatment with a polyclonal antiserum to TGF-β (Colletta *et al*., 1991). Gestodene also inhibited oestrogen-stimulated T47D cell proliferation in sub-lines that had lost their sensitivity to TGF-β to the same extent as in sub-lines that retained their sensitivity to this growth inhibiting factor (Kalkhoven *et al*., 1996); therefore, the involvement of TGF-β in the growth modulating effects of gestodene remains unclear.

Gestodene showed high affinity for and slow dissociation from sex hormone-binding globulin in human serum (Juchem & Pollow, 1990).

When given to female Wistar rats at a dose of 10 mg/kg per day for seven days, gestodene had a slight but significant growth-stimulating effect on the liver, as seen in a 10–15% increase in DNA content without a change in weight (Schulte-Hermann *et al.*, 1988).

4.2.9 *Levonorgestrel* (see also the monograph on 'Hormonal contraceptives, progestogens only', section 4.2.2)

(*a*) *Humans*

In the study of Ruokonen and Käär (1985), described in section 4.2.6, the serum concentrations of ceruloplasmin and cortisol-binding protein were not affected, indicating a lack of oestrogenic activity of levonorgestrel in these women. The serum concentration of sex hormone-binding globulin was markedly decreased, to 50–55% of pre- and post-treatment values at both 30 and 60 days.

Ten women were given 30 μg per day levonorgestrel orally on days 7–10 of the menstrual cycle, and endometrial biopsy samples were taken on the 11th day of the previous cycle and on the day after the last dose (also day 11 of the cycle). Levonorgestrel had no effect on the number of glandular and stromal cell mitoses, basal-cell vacuolation or the diameter and epithelial thickness of the endometrial glands (Landgren *et al.*, 1990).

(*b*) *Experimental systems*

Levonorgestrel binds with high affinity to progesterone receptors (Lemus *et al.*, 1992); its relative binding affinity has been reported to be 1.25 (Kuhnz *et al.*, 1995) to five times (Phillips *et al.*, 1990) higher than that of progesterone itself for the rabbit uterine progesterone receptor and 1.43 and 1.25 times higher for human uterine and recombinant progesterone receptors, respectively (Kuhnz *et al.*, 1995). Metabolites of levonorgestrel showed less or no binding to the progesterone receptor (Lemus *et al.*, 1992).

Levonorgestrel had clear progestational activity *in vivo*, both in a pregnancy maintenance test in female rats (Kuhnz & Beier, 1994) and as measured by inhibition of ovulation and endometrial stimulation in rabbits, indicating that it is slightly less active than progesterone (Phillips *et al.*, 1987).

Levonorgestrel also bound with high affinity to androgen, mineralocorticoid and glucocorticoid receptors (Kloosterboer *et al.*, 1988; Juchem & Pollow, 1990; Phillips *et al.*, 1990), but not to oestrogen receptors (Iqbal *et al.*, 1986; Juchem & Pollow, 1990; Lemus *et al.*, 1992). The relative binding affinity of levonorgestrel for the rat ventral prostate androgen receptor was approximately 20% that of 5α-dihydrotestosterone (Phillips *et al.*, 1990).

Levonorgestrel had moderate androgenic activity *in vivo*, in comparison with 5α-dihydrotestosterone, as measured by stimulation of ventral prostate growth in immature, castrated rats (Phillips *et al.*, 1987; Kuhnz & Beier, 1994).

Levonorgestrel stimulated the growth of oestrogen-sensitive human mammary cancer cells lines. MCF-7 cell proliferation was stimulated by levonorgestrel at a concen-

tration of 10^{-6} mol/L, but only in the presence of insulin added to the medium at ≥ 10 ng/mL (van der Burg *et al.*, 1992). In experiments in which stimulation by levonorgestrel was compared in three sub-lines of MCF-7 and two of T47D cells, stimulation occurred at concentrations of 10^{-7} mol/L and higher in all cell lines except one of the T47D sub-lines (Schoonen *et al.*, 1995a,b). The experiments were performed with breast cancer cell lines grown in phenol red-free medium which contained steroid-free (dextran-coated charcoal-stripped) serum (van der Burg *et al.*, 1992; Schoonen *et al.*, 1995a,b). Under the conditions of these experiments, progesterone receptor expression in both cell types was maintained and was 20–40-fold higher in T47D cells than in MCF-7 cells (van der Burg *et al.*, 1992; Kalkhoven *et al.*, 1994). The stimulating effects of levonorgestrel at 10^{-7}–10^{-6} mol/L were not blocked by simultaneous treatment of the cells with anti-progestogens such as RU486, whereas the anti-oestrogens 4-hydroxytamoxifen (at 10^{-7} mol/L) and ICI164,384 (at 10^{-7}–10^{-6} mol/L) inhibited this stimulation (van der Burg *et al.*, 1992; Schoonen *et al.*, 1995a,b). These findings suggest that the cell proliferation-stimulating effects of levonorgestrel are not mediated via the progesterone receptor but via the oestrogen receptor (Kalkhoven *et al.*, 1994).

Levonorgestrel increased the reductive activity of 17β-hydroxysteroid dehydrogenase in an oestrogen- and progestogen-stimulated MCF-7 cell line in phenol red-free medium. This effect would increase the formation of oestradiol, indicating a possible mechanism by which this progestogen may increase breast cell proliferation *in vivo* (Coldham & James, 1990).

Levonorgestrel had oestrogenic activity at concentrations of 10^{-8}–10^{-6} mol/L when tested for induction of alkaline phosphatase activity as an indicator of oestrogen response in oestrogen receptor-containing and oestrogen-sensitive Ishikawa human endometrial cancer cells (Botella *et al.*, 1995).

Oestradiol at concentrations of 10^{-10} mol/L and higher strongly induced the growth of MCF-7 and T47D cell lines, regardless of the sub-line used (van der Burg *et al.*, 1992; Kalkhoven *et al.*, 1994; Schoonen *et al.*, 1995a,b). The growth stimulation of MCF-7 cells by 10^{-10} mol/L oestrogen was inhibited by 10^{-9} mol/L levonorgestrel in one sub-line, and the effect was not blocked by RU486; no effect was seen in another sub-line (Schoonen *et al.*, 1995a). Oestrogen-induced growth in T47D cells was not blocked by 10^{-6} mol/L levonorgestrel in one sub-line but was totally inhibited in another sub-line at concentrations of 10^{-10}–10^{-8} mol/L. These two T47D sub-lines differ considerably, in that RU486, but not 4-hydroxytamoxifen or ICI164,384, blocked oestrogen-stimulated growth in the former sub-line, while both anti-progestogens and anti-oestrogens inhibited the other (Schoonen *et al.*, 1995b).

Levonorgestrel at concentrations of 0.1–2 ng/mL inhibited the growth of decidual endothelial cells derived from human endometrium, the growth of which was stimulated by exposure to oestradiol at 5 ng/mL but inhibited by lower concentrations and not affected by higher concentrations (Peek *et al.*, 1995).

Levonorgestrel had high affinity for and slow dissociation from sex hormone-binding globulin in human serum (Juchem & Pollow, 1990). It displaced testosterone,

thus at least theoretically resulting in an increase in free testosterone (Nilsson & von Schoultz, 1989).

Protein and mRNA expression of vascular endothelial growth factor was increased in the endometrium of cynomolgus monkeys treated with levonorgestrel for 20 days as compared with endometrial samples from luteal-phase monkeys. These effects were limited to stromal cells for protein expression detected by immunohistochemistry and to the vascular endothelial growth factor-189 isoform for mRNA expression (Greb *et al*., 1997).

Levonorgestrel had no significant effect on the growth of the liver in female Wistar rats (Schulte-Hermann *et al*., 1988).

4.2.10 *Lynoestrenol*

(*a*) *Humans*

Maudelonde *et al*. (1991) studied the effects of lynoestrenol given at a dose of 10 mg/day on days 5–25 of each menstrual cycle for one to three months to 31 pre-menopausal women with biopsy-confirmed benign breast disease, by comparing them with a group of 16 untreated women with similar clinical characteristics. Fine-needle aspirates were obtained at the start of the study and at the end of the one- to three-month treatment. The mean percentage of cells staining positively for oestrogen receptor decreased from about 60 to 20%, while the number of cells staining positively for cathepsin D (as an indicator of oestrogenic activity) remained the same. The pre-treatment values for these two parameters were not significantly different from those found in the untreated controls. The reduction in the number of oestrogen receptor-positive cells was viewed by the authors as consistent with the anti-oestrogenic activity of lynoestrenol.

Ruokonen and Käär (1985) studied the effects of lynoestrenol at a dose of 5 mg/day for 60 days in 30 healthy pre-menopausal women on serum levels of ceruloplasmin and cortisol-binding globulin, as indicators of oestrogenic activity. The concentrations of these two proteins were slightly (10–20%) elevated after 30 and 60 days of treatment as compared with pre-treatment, but this was significant only 30 days after the start of treatment. Nevertheless, the results indicated weak oestrogenic activity of lynoestrenol in these women. The serum concentration of sex hormone-binding globulin was markedly decreased by the treatment, to 60 and 50% of pre-treatment values at 30 and 60 days, respectively.

In the study of Odlind *et al*. (1980), described in section 4.2.3, a dose of 1 mg/day lynoestrenol in combination with 50 μg/day ethinyloestradiol given for the duration of one menstrual cycle increased the concentration of sex hormone-binding globulin by approximately 100%, but a combination with a higher lynoestrenol dose of 2.5 mg/day caused only a non-significant, 17% increase.

(*b*) *Experimental systems*

The progestogen lynoestrenol was found to bind with low affinity to both the oestrogen and the progesterone receptor in rabbit uterine cytosol (Tamaya *et al*., 1977) and to the human endometrial progesterone receptor (Briggs, 1975).

Lynoestrenol has been reported to have oestrogenic activity *in vivo* (Lax, 1987). It enhanced the activity of microsomal oestrone sulfatase in human breast carcinoma tissue, suggesting that it could stimulate the formation of biologically active oestrogen in human breast cancer cells (Prost-Avallet *et al*., 1991).

Lynoestrenol has also been reported to have androgenic properties (Darney, 1995).

4.2.11 *Megestrol acetate*

(*a*) *Humans*

No relevant data were available to the Working Group.

(*b*) *Experimental systems*

The progestogen megestrol acetate has been found to bind strongly to the human uterine progesterone receptor, as determined in a competitive binding assay with 20 000 × *g* supernatants of human endometrium and myometrium (Briggs, 1975). Its 19-nor analogue nomegestrol acetate has very high affinity for the progesterone receptor in rat uterus (Botella *et al*., 1990). Neither megestrol acetate nor nomegestrol acetate affected the growth of the mammary cancer cell lines, MCF-7 and T47D:A18, or *trans*-activated an oestradiol-responsive reporter construct containing oestrogen response elements (Catherino & Jordan, 1995). Nomegestrol acetate also had no oestrogenic activity, as demonstrated by the lack of induction of alkaline phosphatase activity in oestrogen receptor-containing and oestrogen-sensitive Ishikawa human endometrial cancer cells (Botella *et al*., 1995).

In vitro, megestrol acetate stimulated the growth of androgen-sensitive mouse mammary carcinoma Shionogi cells, with a reduction in the doubling time of approximately 50% at a concentration of 10^{-6} mol/L. This effect was counteracted by a 5×10^{-6} mol/L excess of the androgen receptor blocking anti-androgen, hydroxyflutamide, which itself did not stimulate the growth of these cells (Luthy *et al*., 1988). Consistent with these observations, megestrol acetate bound weakly to the rat ventral prostate androgen receptor, with an affinity approximately equal to that of testosterone (Botella *et al*., 1987).

When tested in castrated male rats at a dose of 10 mg given subcutaneously twice daily for 14 days, megestrol acetate increased the ventral prostate weight by about 50% and induced a 13-fold stimulation of the activity of the cell proliferation-related enzyme ornithine decarboxylase in the ventral prostate; similar effects were found with a dose of 0.15 mg 5α-dihydrotestosterone twice daily (Labrie *et al*., 1987). When castrated rats that had received testosterone re-substitution (via silastic implants) were treated with megestrol acetate at 20 mg/kg per day subcutaneously for 14 or 28 days, however, the prostate weights were reduced by 49 and 65%, respectively (Burton & Trachtenberg, 1986). Thus, megestrol acetate has weak androgenic activity in castrated male rats (Labrie *et al*., 1987), while it has clear anti-androgenic activity in intact rats (Burton & Trachtenberg, 1986).

Megestrol acetate bound to the glucocorticoid receptor in human mononuclear leukocytes and induced glucocorticoid-like effects in these cells, including inhibition of proliferative responses to mitogenic stimuli (Kontula *et al*., 1983).

Studies with the human breast cancer cell line ZR-75-1, which contains oestrogen, progesterone and androgen receptors, suggested that megestrol acetate inhibits the growth of these cells through an interaction of androgen and progesterone receptor-mediated mechanisms (Poulin *et al*., 1990).

Megestrol acetate weakly inhibited the induction of angiogenesis by basic fibroblast growth factor and TGF-α in rabbit cornea *in vitro*. This anti-angiogenic activity was not correlated with its binding to glucocorticoid, progesterone or androgen receptors (Yamamoto *et al*., 1994).

4.2.12 *Norethisterone*

(*a*) *Humans*

In the study of Odlind *et al.* (1980), described in section 4.2.3, a dose of 0.5 or 1.0 mg/day norethisterone in combination with 35 μg/day ethinyloestradiol or 50 μg/day mestranol, respectively, given for the duration of one menstrual cycle increased the concentration of sex hormone-binding globulin by approximately 100% in both cases, but the daily dose of 0.5 mg norethisterone in combination with 60 μg ethinyloestradiol given every other day caused only a 45% increase. The dose of 3 mg/day norethisterone acetate in combination with 50 μg/day ethinyloestradiol increased the concentration of sex hormone-binding globulin by approximately 25%.

Ten women were given 300 μg/day norethisterone orally on days 7–10 of the menstrual cycle, and endometrial biopsy samples were taken on the 11th day of the previous cycle and on the day after the last dose (also day 11 of the cycle). The treatment reduced the number of glandular cell mitoses by 65% and markedly increased the number of vacuolated cells in the endometrium, from 0 to 5.5% (Landgren *et al*., 1990).

(*b*) *Experimental systems*

Norethisterone bound with an affinity close to that of the natural ligand to the progesterone receptor in rabbit uterine cytosol (Tamaya *et al*., 1977) and to the nuclear and cytosolic progesterone receptors in human uterine endometrium and myometrium (Briggs, 1975; Shapiro *et al*., 1978; Kasid & Laumas, 1981). It bound with low affinity to the nuclear and the cytosolic progesterone receptors in cultured MCF-7 human breast tumour cells (Kloosterboer *et al*., 1988). In an assay of progestogen-specific stimulation of alkaline phosphatase activity in T47D human breast cancer cells, slightly less than full agonist activity was demonstrated for norethisterone in comparison with progesterone (Markiewicz & Gurpide, 1994). In human endometrial stromal cells in culture, however, norethisterone and progesterone were equally effective in stimulating protein and mRNA expression of insulin-like growth factor binding protein-2 (Giudice *et al*., 1991).

In comparison with progesterone, norethisterone had weak to moderate mixed antagonist/agonist progestational activity; *in vivo* it effectively interfered with pregnancy in the post-nidation period in rats and somewhat less effectively in hamsters, but it also inhibited progesterone-supported pregnancy in ovariectomized rats (Reel *et al*., 1979). Furthermore, it showed weak inhibitory activity on ovulation and endometrial stimu-

lation in rabbits (Phillips *et al*., 1987). In immature female rabbits, norethisterone induced increased expression of uteroglobin in both protein and mRNA (Cerbón *et al*., 1990). This effect is mediated by the progesterone receptor, because it is abolished by RU486 (Pasapera *et al*., 1995).

Norethisterone was found to bind with lower affinity than the natural ligand to the oestrogen receptor in rabbit uterine cytosol (Tamaya *et al*., 1977) and rat uterine homogenate (van Kordelaar *et al*., 1975). Norethisterone acetate inhibited specific binding of oestradiol in the cytosolic fraction of female rat liver at concentrations of 10^{-5}–10^{-4} mol/L, and injection of norethisterone acetate *in vivo* induced nuclear translocation of the oestrogen receptor, i.e. cytosol receptor depletion, in the livers of female rats (Marr *et al*., 1980).

Norethisterone at concentrations of 10^{-7}–10^{-6} mol/L showed weaker oestrogenic activity than oestradiol when tested for its stimulatory effect on alkaline phosphatase activity in Ishikawa human endometrial cancer cells, which is an oestrogen-specific response inhibited by 4-hydroxytamoxifen (Markiewicz *et al*., 1992; Botella *et al*., 1995). Binding of oestradiol to rat uterine oestrogen receptors was reduced by norethisterone both *in vivo* and *in vitro* (Di Carlo *et al*., 1983). In addition, several anti-oestrogenic effects were found *in vivo*: in ovariectomized rats treated subcutaneously with oestradiol valerate at 50 µg/rat once a week, norethisterone acetate at a daily dose of 1 mg was about equally effective as tamoxifen at a daily dose of 0.06 mg/rat in reducing the oestrogen-induced increase in uterine weight and serum prolactin (Spritzer *et al*., 1995). Norethisterone also reduced the hyperplastic response of the uterus in ovariectomized rats after treatment with conjugated equine oestrogen; tamoxifen did not have this effect (Kumasaka *et al*., 1994).

Norethisterone stimulated the growth of most oestrogen-sensitive human mammary cancer cells lines tested (Jeng & Jordan, 1991; Jeng *et al*., 1992; Schoonen *et al*., 1995a,b). It stimulated cell proliferation at concentrations of 10^{-8}–10^{-7} mol/L in studies with the oestrogen receptor-positive MCF-7 and T47D:A18 cell lines (Jeng *et al*., 1992), and these results were confirmed in studies with three sub-lines of MCF-7 and two other T47D cell lines of different origin, except that one of the latter did not respond to norethisterone (Schoonen *et al*., 1995a,b). All of these experiments were performed with cells grown in phenol red-free medium which contained steroid-free (dextran-coated charcoal-stripped) serum (Jeng *et al*., 1992; Schoonen *et al*., 1995a,b). Norethisterone induced *trans*-activation of reporter constructs containing an oestrogen response element coupled to the chloramphenicol acetyltransferase gene transfected into these cells (Jeng *et al*., 1992); however, the cell growth-stimulating and reporter gene *trans*-activating effects of norethisterone at 10^{-6} mol/L were blocked by simultaneous treatment of the cells with the anti-oestrogens 4-hydroxytamoxifen (10^{-7} mol/L) and ICI164,384 (10^{-7}–10^{-6} mol/L), but not by anti-progestogens such as RU486 (Jeng & Jordan, 1991; Jeng *et al*., 1992; Schoonen *et al*., 1995a,b). This suggests that the stimulatory effects of norethisterone on cell proliferation are mediated via the oestrogen receptor, but not the progesterone receptor. Support for this notion was provided by studies indicating that

norethisterone did not stimulate the growth of the oestrogen receptor-negative human breast cancer cell lines MDA-MB-231, BT-20 and T47D:C4 (Jeng *et al*., 1992). Furthermore, the stimulation of MCF-7 cell proliferation by norethisterone was accompanied by a marked decrease in TGF-β2 and TGF-β3 mRNA levels, while the level of TGF-β1 mRNA was not affected. The inhibitory effect on TGF-β2 and TGF-β3 mRNA could be blocked by addition of 4-hydroxytamoxifen (Jeng & Jordan, 1991).

Norethisterone increased the reductive activity of 17β-hydroxysteroid dehydrogenase in an oestrogen- and progestogen-stimulated MCF-7 cell line cultured in the absence of phenol red (Coldham & James, 1990), which indicates that this progestogen stimulates breast cell proliferation *in vivo* by increasing the formation of oestradiol.

Oestradiol at concentrations of 10^{-10} mol/L and higher strongly induced the growth of the MCF-7 and T47D cell lines, regardless of the sub-line used (van der Burg *et al*., 1992; Kalkhoven *et al*., 1994; Schoonen *et al*., 1995a,b). The stimulation of MCF-7 cell growth by oestrogen at 10^{-10} mol/L was not significantly inhibited by norethisterone at the concentrations tested (up to 10^{-6} mol/L) (Schoonen *et al*., 1995a). Oestrogen-induced growth in T47D cells was not blocked by norethisterone at 10^{-6} mol/L in one sub-line (T47D-A), but it was completely inhibited in another sub-line (T47D-S) at a concentration of 10^{-8} mol/L. These two sub-lines differ considerably, in that RU486, but not 4-hydroxytamoxifen or ICI164,384, blocked oestrogen-stimulated growth in the T47D-A cell line, while both anti-progestogens and anti-oestrogens were inhibitory for T47D-S (Schoonen *et al*., 1995b).

In vivo, norethisterone had no androgenic activity, as judged by the lack of stimulation of ventral prostate growth in castrated rats (Phillips *et al*., 1987); however, norethisterone reduced the activity of 5α-reductase in the livers of male and female rats and also decreased the activity of hepatic 3β-hydroxysteroid dehydrogenase in castrated male rats. These effects were not blocked by flutamide or oestradiol, suggesting that androgen receptor-mediation was not involved. The oestrogen-like activity of norethisterone, i.e. the suppression of 3β-hydroxysteroid dehydrogenase, can probably be ascribed to an effect of 'high oestrogen dose' (Lax *et al*., 1984).

Studies with the human breast cancer cell line ZR-75-1, which contains functional oestrogen, progesterone and androgen receptors, suggest that norethisterone inhibits the growth of these cells by a combined action of androgen and progesterone receptor-mediated mechanisms in the presence of oestrogens. In oestrogen-free medium, however, norethisterone stimulated the growth of these cells, an effect that was counteracted by the anti-oestrogen EM-139 (Poulin *et al*., 1990).

Norethisterone did not bind to the glucocorticoid receptor on human mononuclear leukocytes (Kontula *et al*., 1983). It showed moderate affinity for human sex hormone-binding globulin, which could only slightly increase the level of free testosterone (Nilsson & von Schoultz, 1989).

Norethisterone increased secretion of vascular endothelial growth factor by the human breast cancer cell line T47D to a similar extent (two- to threefold over basal levels) as progesterone. This effect, which was progestogen-specific and did not occur in

MCF-7, ZR-75 or MDA-MB-231 cells, suggests an angiogenic response of these cells to norethisterone (Hyder *et al*., 1998).

4.2.13 *Norethynodrel*

(*a*) *Humans*

No relevant data were available to the Working Group.

(*b*) *Experimental systems*

Norethynodrel is metabolized *in vivo* to norethisterone, which binds the progesterone receptor in rabbit uterine cytosol and human uterine endometrium and myometrium, whereas no binding to the progesterone receptor was reported in cultured MCF-7 human breast tumour cells, as pointed out above in the section on norethisterone. Shapiro *et al*. (1978) reported that the binding affinity of norethynodrel itself to the human uterine progesterone receptor is 23% that of progesterone. Progestogen-specific stimulation of alkaline phosphatase activity in T47D human breast cancer cells revealed the full agonist activity of norethynodrel, which was as strong as that of progesterone (Markiewicz & Gurpide, 1994).

The affinity with which norethynodrel bound to the oestrogen receptor in whole rat uterine homogenate was closest to that of the natural ligand of all the progestogens tested (van Kordelaar *et al*., 1975). Norethynodrel at concentrations of 10^{-8}–10^{-6} mol/L showed moderate oestrogenic activity in comparison with oestradiol when tested for its stimulatory effect on alkaline phosphatase activity in Ishikawa human endometrial cancer cells, which is an oestrogen-specific response inhibited by 4-hydroxytamoxifen (Markiewicz *et al*., 1992; Botella *et al*., 1995).

Norethynodrel stimulated the growth of the oestrogen receptor-positive human breast cancer cell lines MCF-7 and T47D-A18 at concentrations of 10^{-8}–10^{-7} mol/L in experiments performed with cells grown in phenol red-free medium which contained steroid-free (dextran-coated charcoal-stripped) serum. Norethynodrel induced *trans*-activation of reporter constructs containing an oestrogen response element coupled to the chloramphenicol acetyltransferase gene transfected into these cells; however, the cell growth-stimulating and reporter gene-*trans*-activating effects of norethynodrel at 10^{-7} mol/L were blocked by simultaneous treatment of the cells with the anti-oestrogens 4-hydroxytamoxifen (10^{-7} mol/L) and ICI164,384 (10^{-7}–10^{-6} mol/L), but not by anti-progestogens such as RU486. These findings suggest that the stimulation of cell proliferation by norethynodrel is mediated via the oestrogen receptor, not the progesterone receptor. Support for this notion is provided by studies indicating that norethynodrel does not stimulate the growth of the oestrogen receptor-negative human breast cancer cell lines MDA-MB-231, BT-20 and T47D:C4 (Jeng *et al*., 1992).

The androgenic activity of norethynodrel has not been studied, but its metabolite norethisterone has androgenic activity *in vivo* (Phillips *et al*., 1987; Duc *et al*., 1995).

Norethynodrel increased the secretion of vascular endothelial growth factor by the human breast cancer cell line T47D to a similar extent (two- to threefold over basal

levels) as progesterone. This effect, which was progestogen-specific and did not occur in MCF-7, ZR-75 or MDA-MB-231 cells, suggests an angiogenic cellular response to norethynodrel (Hyder *et al*., 1998).

The combination of mestranol (7.5 or 75 μg/rat per day) and norethynodrel (0.5 or 5 mg/rat per day) given as a pellet implant to female Sprague-Dawley rats, starting at 45, 55, 65 or 75 days of age, caused changes in the developing mammary gland that resulted in protection against induction of mammary cancer by a single dose (80 mg/kg bw) of DMBA (Russo *et al*., 1989). The hormone treatment was given for 21 days, followed by 21 days' recovery, at which time some rats were killed to study the morphology of their mammary glands, while other rats received DMBA. The hormone treatment at both doses decreased the number of terminal end-buds per mammary gland and increased the number of alveolar buds but did not alter the number of terminal ducts; cell proliferation, measured as the DNA-labelling index, was reduced in the terminal ducts and alveolar buds but remained unchanged in the terminal end-buds (Russo *et al*., 1989; Russo & Russo, 1991). In these experiments, a trend was observed for the hormonal treatment to produce less effect when initiated at a later age. The reduction in cell proliferation in terminal end-buds and terminal ducts, the target tissues for DMBA, may explain the protective effect of the hormonal combination on the development of mammary cancer.

Reboud and Pageaut (1977) administered norethynodrel by subcutaneous implantation of resin pellets to female BALB/C, B6AF1 and C57BL6 mice for two weeks at a dose of 15 mg/mouse once a week and for nine weeks or eight months at 15 mg/mouse every three weeks. Under each of the exposure conditions and in all strains, norethynodrel caused irregular hyperplasia of the vagina and exo-cervix similar to that observed during oestrus, unlike progesterone which caused mucoid and dysplastic cervical changes at the same dose. Progesterone, but not norethynodrel, is a cervical tumour promoter in mice treated with 3-methylcholanthrene.

Norethynodrel had a strong growth-stimulating effect on the livers of female Wistar rats when given at a dose of 10–100 mg/kg bw for seven days, as was evident from a 15–25% increase in liver weight and an approximately 40% increase in hepatic DNA content (Schulte-Hermann *et al*., 1988).

Norethynodrel *per se* at concentrations of 3×10^{-6}–10^{-4} mol/L induced γ-GT activity in cultured rat hepatocytes in the presence of 30 nmol/L dexamethasone (Edwards & Lucas, 1985).

4.2.14 *Norgestimate*

(*a*) *Humans*

No relevant data were available to the Working Group.

(*b*) *Experimental systems*

The binding affinity of norgestimate for human and rabbit uterine progesterone receptors has been reported to be 1–3% that of progesterone itself (Juchem *et al*., 1993; Kuhnz *et al*., 1995). Other studies showed a 10-fold lower (Killinger *et al*., 1985) or even

a somewhat higher binding affinity (Phillips *et al*., 1990) relative to progesterone. The apparent discrepancies in the observed progesterone receptor binding may be due to the fact that metabolites of norgestimate, levonorgestrel (see above) and levonorgestrel-17-acetate, bind with approximately eight- and fourfold higher affinity, respectively, to this receptor in human myometrial tissue (Juchem *et al*., 1993).

Norgestimate had clear progestational activity *in vivo*, both in a test for pregnancy maintenance in female rats (Kuhnz & Beier, 1994) and as measured by inhibition of ovulation and endometrial stimulation in rabbits (Killinger *et al*., 1985; Phillips *et al*., 1987). In these three endocrine bioassays, norgestimate was 3–10 times more active than progesterone (Phillips *et al*., 1987).

In vivo, norgestimate had little or no androgenic activity in comparison with 5α-dihydrotestosterone, as measured by stimulation of ventral prostate growth in immature castrated rats (Phillips *et al*., 1987, 1990; Kuhnz & Beier, 1994).

At concentrations as low as 10^{-10} mol/L, norgestimate up-regulated the expression of the prostate-specific antigen at the mRNA and protein level in T-47D human breast cancer cells. Expression of the antigen in these cells was also stimulated by other progestogens, androgens and corticosteroids (Zarghami *et al*., 1997).

Norgestimate also bound with very low affinity to the androgen receptor but not at all to the oestrogen receptor (Juchem & Pollow, 1990; Phillips *et al*., 1990). The relative binding affinity for the rat ventral prostate androgen receptor was approximately 0.3% that of 5α-dihydrotestosterone (Phillips *et al*., 1990).

Norgestimate did not bind to sex hormone-binding globulin in human serum (Juchem & Pollow, 1990).

4.2.15 *Norgestrel*

(*a*) *Humans*

No relevant data were available to the Working Group.

(*b*) *Experimental systems*

Norgestrel binds strongly to the progesterone receptor in human uterus, with an affinity equal to 50 or > 90% that of progesterone (Briggs, 1975; Shapiro *et al*., 1978). A similar result was reported for receptor binding in the chick oviduct (Haukkamaa *et al*., 1980). Norgestrel bound with sixfold higher affinity than progesterone to the progesterone receptor in rabbit lung (Nielsen *et al*., 1987).

In an assay of progestogen-specific stimulation of alkaline phosphatase activity in T47D human breast cancer cells, moderate agonist activity was demonstrated for norgestrel in comparison with progesterone (Markiewicz & Gurpide, 1994).

Norgestrel at concentrations of 10^{-8}–10^{-6} mol/L stimulated the growth of the oestrogen receptor-containing and oestrogen-sensitive mammary cancer cell lines MCF-7 and T47D:A18; this activity was inhibited by the anti-oestrogens ICI182,780 and ICI164,384 (at a concentration of 10^{-6} mol/L), but not by RU486 (at a concentration of 10^{-7} mol/L) (Jeng *et al*., 1992; Catherino *et al*., 1993; Catherino & Jordan, 1995). Norgestrel did not

affect the growth of the oestrogen receptor-negative and oestrogen-independent mammary cancer cell lines MDA-MB-231, BT-20 and T47DC4 (Jeng *et al*., 1992). Progesterone receptor expression was maintained in both cell types. With reporter constructs containing two progesterone response elements in front of the *tk* promotor coupled to the chloramphenicol acetyltransferase gene transfected into the MCF-7 cell line, transcriptional activation was observed with norgestrel at a concentration of 10^{-6} mol/L, clearly demonstrating expression of functional progesterone. During these experiments, 10^{-10} mol/L oestradiol was present in the medium to boost receptor expression (Catherino *et al*., 1993). These findings suggest that the stimulating effects of norgestrel on cell proliferation are mediated via the oestrogen receptor. This is apparently not related to effects at the level of receptor–ligand interaction, because norgestrel at concentrations of 10^{-9}–10^{-6} mol/L causes *trans*-activation of reporter constructs containing oestrogen response elements (from the vitellogenin or *pS2* gene) or progesterone response elements transfected into MCF-7 cells; ICI164,384, but not RU486, inhibited *trans*-activation of the oestrogen response element-containing constructs, while RU486, but not ICI164,384, inhibited *trans*-activation of the progesterone response element-containing construct (Catherino *et al*., 1993; Catherino & Jordan, 1995). Norgestrel also stimulated the protein expression of the progesterone receptor in MCF-7 cells at 10^{-6} but not at 10^{-8} mol/L (Catherino *et al*., 1993).

Norgestrel showed much weaker binding than the natural ligand to the oestrogen receptor in whole rat-uterine homogenate (van Kordelaar *et al*., 1975), while displacement of ^{3}H-oestradiol binding to the cytosolic fraction of female rat liver occurred only at norgestrel concentrations of 10^{-5}–10^{-4} mol/L (Marr *et al*., 1980). The binding of oestradiol to rat uterine oestrogen receptor was reduced, however, by norgestrel, both *in vivo* at 1 h after a single oral dose of 15 mg/kg bw and *in vitro* (Di Carlo *et al*., 1983). *In vivo*, norgestrel partially reversed the hyperplastic and metaplastic changes found in oestrogen-exposed rat uterus (White *et al*., 1982).

Norgestrel had much weaker oestrogenic activity than oestradiol at concentrations greater than 1×10^{-6} mol/L when tested for its stimulatory effect on alkaline phosphatase activity in Ishikawa human endometrial cancer cells, which is an oestrogen-specific response inhibited by 4-hydroxytamoxifen (Markiewicz *et al*., 1992; Markiewicz & Gurpide, 1994).

Norgestrel was shown to be a potent competitor for binding of 5α-dihydrotestosterone to the androgen receptor in human foreskin fibroblasts, with an activity similar to that of testosterone (Breiner *et al*., 1986).

Studies with the human breast cancer cell line ZR-75-1, which contains functional oestrogen, progesterone and androgen receptors, suggest that norgestrel inhibits the growth of these cells via an interaction of androgen and progesterone receptor-mediated mechanisms in the presence of oestrogens. In oestrogen-free medium, however, norgestrel stimulated the growth of these cells, an effect that was counteracted by the anti-oestrogen EM-139 (Poulin *et al*., 1990).

Norgestrel increased the secretion of vascular endothelial growth factor by the human breast cancer cell line T47D to an extent (two- to threefold over basal levels)

similar to progesterone. This effect, which was progestogen-specific and did not occur in MCF-7, ZR-75 or MDA-MB-231 cells, suggests an angiogenic response of T47D cells to norgestrel (Hyder *et al.*, 1998).

4.3 Genetic and related effects

Most, if not all, of the genetic and related effects associated with use of oral contraceptives can be explained by oestrogen and progestogen receptor mechanisms (King, 1991), but non-receptor processes may also exist (Duval *et al.*, 1983; Yager & Liehr, 1996). The following descriptions indicate how the doses of hormone used relate to receptor and non-receptor mechanisms and to the concentrations achieved *in vivo* in women who use oral contraceptives or post-menopausal hormonal therapy. The concentrations in such formulations are usually several micrograms per kilogram body weight per day, which generate plasma concentrations of nanograms per millilitre for progestogens and picograms per millilitre for oestrogens (Orme *et al.*, 1983; Barnes & Lobo, 1987). Those are the concentrations at which receptor-mediated events can be saturated *in vitro*. Appreciably higher concentrations were used in many of the studies listed in Tables 42–46. The significance of the presence and absence of effects at these concentrations is uncertain, as is the mode of action in the case of effects.

4.3.1 *Combined oral contraceptives*

Genetic changes in cells from women taking steroid hormones have been compared with those in cells from unexposed women in five studies, in all of which few details are given about the hormonal exposure; however, use of oral contraceptives predominated.

Two of three reports described the effects of steroids on lymphocytes. Ghosh and Ghosh (1988) noted an increased frequency of sister chromatid exchange in lymphocytes from 51 healthy, non-smoking Indian women (mean age, 34.5 years) exposed to ethinyloestradiol plus levonorgestrel [doses not given] for 4–28 months as compared with 38 unexposed referents (mean age, 35.6 years). The numbers of sister chromatid exchanges per cell were 5.56 ± 0.21 for the referents and 8.63 ± 0.29 for women taking oral contraceptives ($p < 0.001$).

In contrast, a study in Denmark showed no effect on the sister chromatid exchange frequency in lymphocytes of exposure to oestrogen and progestogen [types and doses not stated] for a minimum of two months (Husum *et al.*, 1982). There were 25 non-smoking, healthy women aged 15–42 years in the referent group, who had 8.42 ± 0.21 sister chromatid exchanges per cell and 15 women with otherwise similar characteristics who used oral contraceptives and had 8.54 ± 0.24 sister chromatid exchanges per cell. Smoking of > 20 cigarettes per day produced the expected increase in sister chromatid exchange frequency, but no significant difference was observed between oral contraceptive users who smoked this number of cigarettes and comparable controls: 9.52 ± 0.30 sister chromatid exchanges per cell in 13 referents and 10.36 ± 0.75 sister chromatid exchanges per cell in six oral contraceptive users.

Chromosomal abnormalities were quantified in lymphocytes from 88 women aged 16–35 years in South Africa, equally divided into controls who had never used hormonal contraception and women who had used oral contraceptives [types and doses not stated] for 7–98 months. The groups were pair-matched for race, age, parity and condition of offspring, occupation, medication, X-irradiation and smoking habits (Pinto, 1986). Abnormal chromosomes were found in 31% (410/1286) of lymphocytes from oral contraceptive users and 18% (233/1255) of control cells ($p < 0.0001$). The abnormalities were subclassified into those possibly caused by technical handling (0.29 ± 0.13 and 0.19 ± 0.07 abnormalities per cell in oral contraceptive users and controls, respectively ($p < 0.0001$)) and those not likely to be generated in this way (0.105 ± 0.077 and 0.018 ± 0.029 abnormalities per cell in oral contraceptive users and controls, respectively ($p < 0.0001$)).

Indications of hormone-related genetic damage in lymphocytes in two of the three well-conducted studies raised questions about the potential genotoxicity of steroid hormones in humans. As the relevance of effects in blood lymphocytes to mechanisms of carcinogenesis in tissues such as breast epithelium is unclear, two good analyses of the effects of oral contraceptives on subsequent changes in breast cancer DNA are noteworthy. The two studies were based on the same library of stored breast cancer tissues from women in Sweden whose previous exposure to oral contraceptives was known. At the time of first diagnosis of the cancer, pre-menopausal women were questioned about their earlier life style, including the age at which they had started using oral contraceptives. Tumours removed from these women were stored and subsequently used to analyse ploidy, aneuploidy and cell proliferative activity by flow cytometry (Olsson *et al*., 1991b) and oncogene amplification (Olsson *et al*., 1991c).

In the study of ploidy (Olsson *et al*., 1991b), 175 breast tumours from pre-menopausal women aged 26–52 years were used. Of the tumours from women who had started using oral contraceptives before 20 years of age, 81% ($n = 27$) were aneuploid, whereas only 53% ($n = 59$) of those from women who had never used oral contraceptives were aneuploid ($p < 0.04$). Tumours from women who had started using oral contraceptives at ages 20 to ≥ 24 years had intermediate percentages of aneuploid cells. There was a highly significant ($p = 0.0001$) correlation between early oral contraceptive use and age at diagnosis and other parameters such as proliferative activity, measured as the fraction of cells in S-phase. The statistical significance of the association between early oral contraceptive use and biological effects on the cancer cells was maintained when multivariate analysis was performed.

In the study of oncogenes (Olsson *et al*., 1991c), *erbb*2 (*HER*/neu) and *int*2 gene amplifications were assessed in 72 tumours from 28–50-year-old women. More cancers from women who had started using oral contraceptives before the age of 20 had *erbb*2 amplifications (11/19 or 58% of cancers) than those from women who started after that age (11/53 or 21% of cancers). The odds ratio for this difference was significant in both univariate (odds ratio, 5.3; 95% CI, 1.6–17) and multivariate (odds ratio, 6.8; 95% CI, 1.3–35) analyses. No link was seen between early oral contraceptive use and *int*2 amplification, but this effect was positively associated with any use of progestogens (multivariate

odds ratio, 17; 95% CI, 1.8–170); amplification of *erbb*2 was not related to progestogen use. Other variables considered were age at abortion and first full-term pregnancy, parity, age at diagnosis and tumour stage. The authors recognized the problems associated with interpreting data from such analyses in terms of cause and effect and correctly concluded that they should not be ignored.

4.3.2 *Ethinyloestradiol and some derivatives alone and in combination with progestogens* (Table 42)

No gene mutation was induced in *S. typhimurium* after treatment with ethinyloestradiol. In single studies, small increases in the frequency of unscheduled DNA synthesis were demonstrated in primary cultures of rat hepatocytes treated with ethinyloestradiol, particularly in cells from male rats, and cell transformation was demonstrated *in vitro* in BALB/c 3T3 mouse cells treated with ethinyloestradiol.

In vivo, covalent binding to DNA was demonstrated in the liver, pancreas and kidney of rats and in the kidneys of Syrian hamsters treated with ethinyloestradiol. Chromosomal aberrations were induced *in vivo* in kidney cells of exposed, castrated male Syrian hamsters and in bone-marrow cells of mice treated with high doses of ethinyloestradiol. Paradoxically, ethinyloestradiol at these high doses did not induce micronuclei in bone-marrow cells of mice.

Covalent binding to DNA was observed in the kidneys of Syrian hamsters treated with ethylethinyloestradiol and in those of animals that had received an implant of methylethinyloestradiol. Methoxyethinyloestradiol (Moxestrol) did not induce cell transformation in BALB/c 3T3 mouse cells *in vitro*, but it bound to DNA in the kidneys of Syrian hamsters that had received a Moxestrol implant.

Combinations of ethinyloestradiol and gestodene did not induce gene mutation in various *S. typhimurium* strains. Chromosomal aberrations were induced in the bone marrow of male mice treated *in vivo* with combinations of ethinyloestradiol and norethisterone acetate. In a single study, micronuclei were not induced in female mice exposed *in vivo* to ethinyloestradiol and norethisterone acetate. Primary cultures of baby rat kidney cells were transformed to anchorage-independent growth, and these cells induced tumour formation in syngeneic animals infected with HPV-16 DNA and Ha-*ras*-1 and exposed to an ethanolic extract of oral contraceptive tablets containing ethinyloestradiol and levonorgestrel.

DNA covalent binding was demonstrated in the liver, pancreas and kidney of male rats treated with ethinyloestradiol and tamoxifen.

4.3.3 *Mestranol alone and in combination with progestogens* (Table 43)

Gene mutations were not induced in *S. typhimurium* after treatment with mestranol itself or with ethanolic extracts of Ovulen 21 tablets, containing mestranol, or Enovid tablets, containing mestranol and norethynodrel. Also, gene mutations were not induced in *S. typhimurium* in a host-mediated assay in which the bacteria were recovered from the livers of mice. It has been reported, however, that mestranol and extracts of Ovulen 21 and

Table 42. Genetic and related effects of ethinyloestradiol and its derivatives

Test system	Result[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Ethinyloestradiol				
Escherichia coli rec strains, differential toxicity	NT	–	1000 µg/plate	Mamber *et al*. (1983)
Bacillus subtilis rec strains, differential toxicity	–	NT	5000 µg/plate	Tanooka (1977)
Salmonella typhimurium TA100, TA1535, TA1537, TA1538, TA98, reverse mutation	–	–	2500 µg/plate	Lang & Redmann (1979)
Salmonella typhimurium TA100, TA1535, TA1537, TA1538, TA98, reverse mutation	–	–	1000 µg/plate	Dayan *et al*. (1980)
Salmonella typhimurium TA100, TA1535, TA1537, TA1538, TA98, reverse mutation	–	–	500 µg/plate[c]	Lang & Reimann (1993)
Aneuploidy, male Chinese hamster DON cells *in vitro*	+	NT	22.2	Wheeler *et al*. (1986)
Gene mutation, Chinese hamster lung V79 cells, *hprt* locus *in vitro*	–	–	29.6	Drevon *et al*. (1981)
Gene mutation, Chinese hamster lung V79 cells, ouabain resistance *in vitro*	–	–	29.6	Drevon *et al*. (1981)
Chromosomal aberrations, Chinese hamster ovary cells *in vitro*	–	NT	4	Ishidate *et al*. (1978)
Cell transformation, BALB/c 3T3 mouse cells	–	NT	5.0	Dunkel *et al*. (1981)
Cell transformation, BALB/c 3T3 mouse cells	+	NT	3.0	Liehr *et al*. (1987a)
Cell transformation, Syrian hamster embryo cells, clonal assay	–	NT	50	Dunkel *et al*. (1981)
Cell transformation, RMuLV/Fischer rat embryo cells	+	NT	10.7	Dunkel *et al*. (1981)
Chromosomal aberrations, human lymphocytes *in vitro*	–	NT	100	Stenchever *et al*. (1969)
Micronucleus induction, mice *in vivo*	–		0.2 po × 15	Shyama & Rahiman (1996)
Chromosomal aberrations, male Syrian hamster kidney cells *in vivo*	+		185 µg/d imp.; 5 mo	Banerjee *et al*. (1994)
Chromosomal aberrations, mouse bone-marrow cells *in vivo*	+		0.12 po × 15	Shyama & Rahiman (1996)
Binding (covalent) to DNA, female rat liver, pancreas, kidney *in vivo*	+		75 µg/d po; 12 mo	Shimomura *et al*. (1992)
Binding (covalent) to DNA, Syrian hamster kidney *in vivo*	+		22 mg imp. × 2	Liehr *et al*. (1987b)
Inhibition of metabolic cooperation, Chinese hamster V79 cells *in vitro*	(+)	NT	0.74	Yager (1983)

Table 42 (contd)

Test system	Result[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Ethinyloestradiol + gestodene (1 part + 2.5 parts)				
Salmonella typhimurium TA100, TA1535, TA1537, TA1538, TA98, reverse mutation	–	–	2000 µg/plate[c,d]	Lang & Reimann (1993)
Ethinyloestradiol + norethisterone acetate				
Chromosomal aberrations, female Swiss mouse bone-marrow cells *in vivo*	+		0.8 po × 15[d]	Shyama *et al.* (1991)
Micronucleus formation, female Swiss mouse bone-marrow cells *in vivo*	–		8.0 po × 15[d]	Shyama *et al.* (1991)
Ethinyloestradiol + (l)-norgestrel				
Cell transformation, primary baby rat kidney+ HPV-16 + H-*ras*-1	+	NT	0.3 (ethanol extract)[d]	Pater *et al.* (1990)
Ethinyloestradiol + tamoxifen				
Binding (covalent) to DNA, female rat liver, pancreas, kidney *in vivo*	+		75 µg + 500 µg/d po, 12 mo	Shimomura *et al.* (1992)
Ethylethinyloestradiol				
Binding (covalent) to DNA, Syrian hamster kidney *in vivo*	+		25 mg imp. × 2	Liehr *et al.* (1987b)
Methoxyethinyloestradiol (Moxestrol)				
Cell transformation, BALB/c 3T3 mouse cells *in vitro*	–	NT	16.3	Liehr *et al.* (1987a)
Binding (covalent) to DNA, Syrian hamster kidney *in vivo*	+		25 mg imp. × 2	Liehr *et al.* (1986)
Methylethinyloestradiol				
Binding (covalent) to DNA, Syrian hamster kidney *in vivo*	+		25 mg imp. × 2	Liehr *et al.* (1986, 1987b)

[a] +, positive; (+), weak positive; –, negative; NT, not tested; ?, inconclusive
[b] LED, lowest effective dose; HID, highest ineffective dose; in-vitro tests, µg/mL; in-vivo tests, mg/kg bw per day; po, oral; imp., implant; d, day; mo, month
[c] Toxicity was observed at higher dose(s) tested
[d] Total mixture

Table 43. Genetic and related effects of mestranol

Test system	Result[b]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Mestranol				
Salmonella typhimurium TA100, TA1535, TA1537, TA1538, TA98, reverse mutation	–	–	12.5 μg/plate	Rao *et al*. (1983)
Salmonella typhimurium TA100, TA1535, TA97a, TA98, reverse mutation	–	–	1000 μg/plate[c]	Dhillon *et al*. (1994)
Drosophila melanogaster, sex-linked recessive lethal mutations	–		50000	Aguiar & Tordecilla (1984)
Sister chromatid exchange, human lymphocytes *in vitro*	+	NT	1	Dhillon *et al*. (1994)
Chromosomal aberrations, human lymphocytes *in vitro*	–	NT	10	Stenchever *et al*. (1969)
Chromosomal aberrations, human lymphocytes *in vitro*	+	NT	100	Dhillon *et al*. (1994)
DNA strand breaks, cross-links or related damage, Sprague-Dawley rats *in vivo*	–		250 ip × 1	Yager & Fifield (1982)
Chromosomal aberrations, mouse bone-marrow cells *in vivo*	–		100 po × 1	Ansari & Adhami (1977)
Chromosomal aberrations, mouse bone-marrow cells *in vivo*	+		0.01 ip × 1	Dhillon *et al*. (1994)
Micronucleus induction, mouse bone-marrow cells *in vivo*	+		1 ip × 1	Dhillon *et al*. (1994)
Sister chromatid exchange, mouse bone-marrow cells *in vivo*	+		0.1 ip × 1	Dhillon *et al*. (1994)
Host mediated assay (male Swiss albino mouse, intravenous inoculation), *S. typhimurium* TA100, TA1535, TA98, TA97a	–		100 ip × 1	Dhillon *et al*. (1994)
Inhibition of metabolic cooperation, Chinese hamster V79 cells *in vitro*	(+)	NT	0.78	Yager (1983)
Mestranol + 2-acetylaminofluorene (3 μg)				
Salmonella typhimurium TA100, TA98, reverse mutation	NT	+	0.62 μg/plate	Rao *et al*. (1983)
Mestranol + norethisterone				
Sister chromatid exchange, human lymphocytes *in vitro*	–	NT	0.0038 + 0.075	Dutkowski *et al*. (1983)
Micronucleus induction, human lymphocytes *in vitro*	–	NT	0.0038 + 0.075	Dutkowski *et al*. (1983)

Table 43 (contd)

Test system	Result[b]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Enovid extract (mestranol + norethynodrel)				
Salmonella typhimurium TA100, TA98, reverse mutation	–	–	625 µg/plate	Rao *et al*. (1983)
Enovid extract (mestranol + norethynodrel) + 2-acetylaminofluorene (3 µg)				
Salmonella typhimurium TA100, TA98, reverse mutation	–	+	31.3 µg/plate	Rao *et al*. (1983)
Enovid extract (mestranol + norethynodrel) + *N*-nitrosopiperidine (250 µg)				
Salmonella typhimurium TA100, reverse mutation	–	+	62.5 µg/plate	Rao *et al*. (1983)
Salmonella typhimurium TA1535, reverse mutation	–	+	31.3 µg/plate	Rao *et al*. (1983)
Ovulen 21 extract (mestranol)				
Salmonella typhimurium TA100, TA98, reverse mutation	–	–	50 µg/plate	Rao *et al*. (1983)
Ovulen 21 extract (mestranol) + 2-acetylaminofluorene (3 µg)				
Salmonella typhimurium TA98, reverse mutation	–	+	31.3 µg/plate	Rao *et al*. (1983)
Ovulen 21 extract (mestranol) + *N*-nitrosopiperidine (250 µg)				
Salmonella typhimurium TA1535, reverse mutation	–	+	31.3 µg/plate	Rao *et al*. (1983)

[a] +, positive; (+), weak positive; –, negative; NT, not tested
[b] LED, lowest effective dose; HID, highest ineffective dose; in-vitro tests, µg/mL; in-vivo tests, mg/kg bw per day; ip, intraperitoneal; po, oral
[c] Toxicity was observed at higher dose(s)

Enovid enhanced the mutation yield obtained with an ineffective dose of 2-acetylaminofluorene (3 μg/plate). The extracts also enhanced the mutation yield obtained with an ineffective dose of *N*-nitrosopiperidine (250 μg/plate).

In a single study, mestranol induced sister chromatid exchange and chromosomal aberrations in human lymphocytes *in vitro* and sister chromatid exchange, chromosomal aberrations and micronuclei in bone-marrow cells from mice treated *in vivo*.

Negative results were obtained with a combination of mestranol and norethisterone in a study of sister chromatid exchange and micronucleus formation in human lymphocyte cultures. The concentrations of the test material used in this study were in the nanogram per millilitre range, which are those that might be expected during human use.

4.3.4 *Cyproterone acetate, metabolites and derivatives* (Table 44)

Gene mutations were not induced in *Salmonella typhimurium* by cyproterone acetate or 6,7-epoxycyproterone acetate. Covalent binding to DNA was observed with cyproterone acetate in cultured rat liver cells, the binding being greater in cells from females than from males in all cases, according to one study. Cyproterone acetate also bound to DNA in human and porcine hepatocytes in culture, while a metabolite, 3-hydroxycyproterone acetate, and a derivative, 3-*O*-acetylcyproterone acetate, also bound to isolated calf thymus DNA. DNA strand breakage was induced in female rat hepatocytes *in vitro* in one study. No DNA breakage was observed in male human hepatocytes *in vitro*. DNA repair, including unscheduled DNA synthesis, of damage induced by cyproterone acetate appears to be sex specific, since these processes occur in cultured liver cells from female but not male rats. Gene mutations were not induced at the *hprt* locus of Chinese hamster V79 cells by cyproterone acetate in two studies; in one of the studies, the cells were co-cultured with rat hepatocytes. The frequency of chromosomal aberrations was not increased in a single study with cyproterone acetate in Chinese hamster V79 cells co-cultured with rat hepatocytes, whereas a study of the frequency of micronucleus formation *in vitro* in hepatocytes from female rats gave inconclusive results.

In vivo, covalent binding to DNA has been demonstrated in the livers of male and female rats and female mice (weak binding). No binding to male mouse liver DNA was observed. Unscheduled DNA synthesis was induced in one study in hepatocytes from rats exposed to cyproterone acetate. Also in single studies, this compound increased the frequency of micronucleus formation in hepatocytes from exposed female rats and induced γ-GT-positive foci in the livers of female rats. In one study, the frequency of mutation of the *Lac*I transgene was significantly increased in female BigBlue® transgenic Fischer 344 rats after exposure to 3-*O*-acetylcyproterone acetate. DNA adducts were quantified in the same experiment; the mutation frequency started to increase at doses at which the number of DNA adducts had already reached a plateau.

4.3.5 *Norethisterone alone and in combination with an oestrogen* (Table 45)

Gene mutation was not induced in *S. typhimurium* after treatment with norethisterone. Chromosomal aberrations were induced, but the frequency of micronucleus

Table 44. Genetic and related effects of cyproterone acetate and some derivatives

Test system	Result[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Cyproterone acetate				
Salmonella typhimurium TA100, TA1535, TA1537, TA1538, TA98, reverse mutation	–	–	250 µg/plate[c]	Lang & Reimann (1993)
DNA strand breaks, alkaline elution assay, female rat hepatocytes *in vitro*	+	NT	20.85	Martelli *et al.* (1995)
DNA repair exclusive of unscheduled DNA synthesis, female rat hepatocytes *in vitro*	+	NT	0.83	Neumann *et al.* (1992)
DNA repair exclusive of unscheduled DNA synthesis, female rat hepatocytes *in vitro*	+	NT	0.83	Topinka *et al.* (1995)
DNA repair exclusive of unscheduled DNA synthesis, male rat hepatocytes *in vitro*	–	NT	20.9	Topinka *et al.* (1995)
Unscheduled DNA synthesis, female rat hepatocytes *in vitro*	+	NT	1.32	Kasper *et al.* (1995)
Unscheduled DNA synthesis, male rat hepatocytes *in vitro*	–	NT	20.85	Martelli *et al.* (1995)
Unscheduled DNA synthesis, female rat hepatocytes *in vitro*	+	NT	0.42	Martelli *et al.* (1995)
Unscheduled DNA synthesis, female rat hepatocytes *in vitro*	+	NT	0.84	Martelli *et al.* (1996a)
Gene mutation,Chinese hamster V79 cells, *hprt* locus *in vitro*	–	–	80[d]	Lang & Reimann (1993)
Gene mutation, Chinese hamster V79 cells, *hprt* locus *in vitro* (co-cultured with hepatocytes)	NT	–	41.7	Kasper *et al.* (1995)
Chromosomal aberrations, Chinese hamster V79 cells *in vitro* (co-cultured with hepatocytes)	NT	–	41.7	Kasper *et al.* (1995)
Micronucleus formation, female rat hepatocytes *in vitro*	(+)	NT	0.42	Kasper *et al.* (1995)
DNA strand breaks, alkaline elution assay, male human hepatocytes *in vitro*	–	NT	20.85 (1 sample)	Martelli *et al.* (1995)
DNA strand breaks, alkaline elution assay, female human hepatocytes *in vitro*	(+)	NT	20.8 (3/4 samples)	Martelli *et al.* (1995)
Unscheduled DNA synthesis, male and female human hepatocytes *in vitro*	+	NT	0.42	Martelli *et al.* (1995)
Unscheduled DNA synthesis, male and female human hepatocytes *in vitro*	+	NT	0.42	Martelli *et al.* (1996a)

Table 44 (contd)

Test system	Result[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Cyproterone acetate (contd)				
Unscheduled DNA synthesis, female rat hepatocytes *in vivo*	+		100 po × 1	Kasper & Mueller (1996)
Gene mutation, female *lacI* transgenic rat (BigBlue®) *in vivo*	+		75 po × 1	Krebs *et al*. (1998)
Micronucleus formation, female rat hepatocytes *in vivo*	+		100 po × 1	Martelli *et al*. (1996b)
γ-Glutamyl transpeptidase-positive foci, female Sprague-Dawley rat liver *in vivo*	+		100 po × 6 (weekly)	Martelli *et al*. (1996b)
Binding (covalent) to DNA, female rat hepatocytes *in vitro*	+	NT	0.013	Topinka *et al*. (1993, 1995)
Binding (covalent) to DNA, male rat hepatocytes *in vitro*	+	NT	0.42	Topinka *et al*. (1993, 1995)
Binding (covalent) to DNA, human (male and female), rat (female) hepatocytes *in vitro*	+	NT	4.2	Werner *et al*. (1996)
Binding (covalent) to DNA, pig (male and female), rat (male) hepatocytes *in vitro*	(+)	NT	4.2	Werner *et al*. (1996)
Binding (covalent) to DNA, female rat liver *in vivo*	+		0.1 po × 1	Topinka *et al*. (1993)
Binding (covalent) to DNA, male rat liver *in vivo*	+		3 po × 1	Topinka *et al*. (1993)
Binding (covalent) to DNA, female rat liver *in vivo*	+		10 po × 1	Werner *et al*. (1995)
Binding (covalent) to DNA, male rat liver *in vivo*	+		100 po × 1	Werner *et al*. (1995)
Binding (covalent) to DNA, male rat liver *in vivo*	+		100 po × 1	Werner *et al*. (1996)
Binding (covalent) to DNA, male C57BL/6 mouse liver *in vivo*	–		35 po × 1	Werner *et al*. (1996)
Binding (covalent) to DNA, female rat liver *in vivo*	+		10 po × 1	Werner *et al*. (1996)
Binding (covalent) to DNA, female C57BL/6 mouse liver *in vivo*	(+)		35 po × 1	Werner *et al*. (1996)
Binding (covalent) to DNA, female rat liver *in vivo*	+		25 po × 1	Krebs *et al*. (1998)

Table 44 (contd)

Test system	Result[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
6,7-Epoxycyproterone acetate				
Salmonella typhimurium TA100, TA1535, TA1537, TA1538, TA98, reverse mutation	–	–	500 µg/plate[c]	Lang & Reimann (1993)
α-Hydroxycyproterone acetate				
Binding (covalent) to DNA, calf thymus *in vitro*	+	NT	1	Kerdar *et al*. (1995)
3-*O*-Acetyl cyproterone acetate (not a metabolite)				
Gene mutation, female *lacI* transgenic rat (BigBlue®) *in vivo*	+		75 po × 1	Krebs *et al*. (1998)
Binding (covalent) to DNA, calf thymus *in vitro*	+	NT	1	Kerdar *et al*. (1995)
Binding (covalent) to DNA, female rat liver *in vivo*	+		25 po × 1	Krebs *et al*. (1998)

[a] +, positive; (+), weak positive; –, negative; NT, not tested
[b] LED, lowest effective dose; HID, highest ineffective dose; in-vitro tests, µg/mL; in-vivo tests, mg/kg bw per day; po, oral
[c] Toxicity was observed at this dose in some or all strains
[d] Some toxicity was observed

Table 45. Genetic and related effects of norethisterone and its ester

Test system	Result[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Norethisterone				
Salmonella typhimurium TA100, TA1535, TA1537, TA98, reverse mutation	–	–	1000 µg/plate	Peter *et al*. (1981)
Salmonella typhimurium TA100, TA1535, TA1537, TA1538, TA98, reverse mutation	–	–	750 µg/plate	Lang & Reimann (1993)
Unscheduled DNA synthesis, female rat hepatocytes *in vitro*	–	NT	150	Blakey & White (1985)
Unscheduled DNA synthesis, male rat hepatocytes *in vitro*	(+)	NT	15	Blakey & White (1985)
Micronucleus formation, female Swiss albino mouse bone-marrow cells *in vivo*	–		30 po × 15	Shyama & Rahiman (1993)
Chromosomal aberrations, female Swiss albino mouse bone-marrow cells *in vivo*	–		70 po × 1	Ansari & Adhami (1977)
Chromosomal aberrations, female Swiss albino mouse bone-marrow cells *in vivo*	+		3 po × 15	Shyama & Rahiman (1993)
Norethisterone acetate				
Salmonella typhimurium TA100, TA1535, TA1537, TA1538, TA98, reverse mutation	–	–	2500 µg/plate	Lang & Redmann (1979)
Salmonella typhimurium TA100, TA1535, TA1537, TA1538, TA98, reverse mutation	–	–	1000 µg/plate	Dayan *et al*. (1980)
Salmonella typhimurium TA100, TA1535, TA1537, TA1538, TA98, reverse mutation	–	–	500 µg/plate[c]	Lang & Reimann (1993)
Salmonella typhimurium TA100, TA1535, TA97a, TA98, reverse mutation	–	–	1000 µg/plate[c]	Dhillon & Dhillon (1996)
Sister chromatid exchange, human male lymphocytes *in vitro* (24-h treatment)	+	NT	1	Dhillon & Dhillon (1996)
Sister chromatid exchange, human male lymphocytes *in vitro* (90-min treatment)	+	+	1	Dhillon & Dhillon (1996)
Chromosomal aberrations, human lymphocytes *in vitro*	–	NT	100	Stenchever *et al*. (1969)
Chromosomal aberrations, human male lymphocytes *in vitro* (72-h treatment)	+	NT	1	Dhillon & Dhillon (1996)
Chromosomal aberrations, human male lymphocytes *in vitro* (6-h treatment)	–	+	10	Dhillon & Dhillon (1996)
Host-mediated assay, male Swiss albino mouse (intravenous inoculation), *S. typhimurium* TA97a, TA98, TA100, TA1535	–		100 ip × 1	Dhillon & Dhillon (1996)
Sister chromatid exchange, Swiss albino mice bone-marrow cells *in vivo*	+		1 ip × 1	Dhillon & Dhillon (1996)
Micronucleus formation, male Swiss albino mouse bone-marrow cells *in vivo*	+		1 ip × 1	Dhillon & Dhillon (1996)
Dominant lethal mutation induction, C3H and NMRI mice *in vivo*	(+)		1 mg/animal po daily × 4 wk	Rohrborn & Hansmann (1974)
Aneuploidy, C3H mice *in vivo*	+		10 mg/animal po daily × 4 wk	Rohrborn & Hansmann (1974)

Table 45 (contd)

Test system	Result[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Norethisterone + mestranol				
Sister chromatid exchange, human lymphocytes *in vitro*	–	NT	0.075 + 0.0038	Dutkowski *et al*. (1983)
Micronucleus formation, human lymphocytes *in vitro*	–	NT	0.075 + 0.0038	Dutkowski *et al*. (1983)
Norethisterone acetate + ethinyloestradiol				
Chromosomal aberrations, female Swiss albino mouse bone-marrow cells *in vivo*	+		0.79 + 0.01 po × 15	Shyama *et al*. (1991)
Micronucleus formation, female Swiss albino mouse bone-marrow cells *in vivo*	–		7.9 + 0.1 po × 15	Shyama *et al*. (1991)

[a] +, positive; (+), weak positive; –, negative; NT, not tested
[b] LED, lowest effective dose; HID, highest ineffective dose; in-vitro tests, μg/mL; in-vivo tests, mg/kg bw per day; po, oral; ip, intraperitoneal; wk, week
[c] Precipitation and/or toxicity was observed at higher dose(s).

formation was not increased in bone-marrow cells of female Swiss albino mice treated *in vivo* with norethisterone.

In a single study, the frequencies of sister chromatid exchange and micronuclei were not increased in cultured human lymphocytes treated with a combination of norethisterone and mestranol.

4.3.6 *Norethisterone acetate alone and in combination with an oestrogen* (Table 45)

Norethisterone acetate did not induce gene mutation in various strains of *S. typhimurium* with or without an exogenous metabolic activation system. In a single study, the frequencies of sister chromatid exchange and chromosomal aberrations were increased in human lymphocytes treated *in vitro* with norethisterone acetate. In the same study, sister chromatid exchange and micronucleus formation were induced in the bone-marrow cells of Swiss albino mice treated *in vivo* with norethisterone acetate, whereas gene mutations were not induced in *S. typhimurium* in a mouse host-mediated assay in which the bacteria were recovered from the livers of animals after treatment with norethisterone acetate.

The combination of norethisterone acetate plus ethinyloestradiol induced chromosomal aberrations in bone-marrow cells of female Swiss albino mice exposed *in vivo*, whereas micronuclei were not induced in the bone-marrow cells of these mice.

4.3.7 *Chlormadinone acetate* (Table 46)

After rat liver cells were incubated with chlormadinone acetate *in vitro*, covalent DNA binding was observed, particularly in cells from females. Unscheduled DNA synthesis was reported in female but not male rat hepatocytes *in vitro* and in male and female human hepatocytes *in vitro* after treatment with chlormadinone acetate. The BdUr density shift assay to determine DNA repair in female and male rat hepatocytes exposed to chlormadinone acetate *in vitro* gave negative results.

No DNA binding was observed in female rat liver *in vivo*, but micronuclei were induced in female rat hepatocytes *in vivo*.

4.3.8 *Gestodene* (Table 46)

Gene mutations were not induced in *S. typhimurium* by gestodene or combinations of gestodene and ethinyloestradiol.

4.3.9 *Megestrol acetate* (Table 46)

Low levels of covalent DNA binding were observed in rat liver cells treated with megestrol acetate *in vitro*, particularly in cells from females. Megestrol acetate induced unscheduled DNA synthesis in rat primary hepatocytes and gave rise to DNA repair in female and male rat liver cells and in human hepatocytes *in vitro*.

Weak covalent DNA binding was observed in female rat liver *in vivo*; however, micronucleus formation was not induced in female rat hepatocytes, and γ-GT-positive foci were not induced in rat liver *in vivo*.

Table 46. Genetic and related effects of other progestogens used in combined oral contraceptives

Test system	Result[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Chlormadinone acetate				
Salmonella typhimurium TA100, TA1535, TA1537, TA1538, reverse mutation	–	–	1000 µg/plate	Dayan *et al*. (1980)
DNA repair exclusive of unscheduled DNA synthesis, female and male rat hepatocytes *in vitro*	–	NT	20.3	Topinka *et al*. (1995)
Unscheduled DNA synthesis, female rat hepatocytes *in vitro*	+	NT	0.81	Martelli *et al*. (1996a)
Unscheduled DNA synthesis, male rat hepatocytes *in vitro*	–	NT	8.1	Martelli *et al*. (1996a)
Cell transformation, rat liver cells treated *in vivo* scored *in vitro*	–		100 po × 6	Martelli *et al*. (1996b)
Unscheduled DNA synthesis, male and female human hepatocytes *in vitro*	+	NT	0.81	Martelli *et al*. (1996a)
Chromosomal aberrations, human lymphocytes *in vitro*	–	NT	100	Stenchever *et al*. (1969)
Micronucleus formation, female rat hepatocytes *in vivo*	+		100 po × 1	Martelli *et al*. (1996b)
Binding (covalent) to DNA, female rat hepatocytes *in vitro*	+	NT	1.2	Topinka *et al*. (1995)
Binding (covalent) to DNA, male rat hepatocytes *in vitro*	(+)	NT	1.2	Topinka *et al*. (1995)
Binding (covalent) to DNA, female rat liver *in vivo*	–		100 po × 1	Topinka *et al*. (1995)
Gestodene				
Salmonella typhimurium TA100, TA1535, TA1537, TA1538, TA98, reverse mutation	–	–	5 µg/plate	Lang & Reimann (1993)
Gestodene + ethinyloestradiol (2.5 parts + 1 part)				
Salmonella typhimurium TA100, TA1535, TA1537, TA1538, TA98, reverse mutation	–	–	200 µg/plate[c,d]	Lang & Reimann (1993)
Megestrol acetate				
DNA repair exclusive of unscheduled DNA synthesis, female and male rat hepatocytes *in vitro*	–	NT	19.3	Topinka *et al*. (1995)
Unscheduled DNA synthesis, female rat hepatocytes *in vitro*	+	NT	1.93	Martelli *et al*. (1996a)

Table 46 (contd)

Test system	Result[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Megestrol acetate (contd)				
Unscheduled DNA synthesis, male rat hepatocytes *in vitro*	–	NT	19.3	Martelli *et al*. (1996a)
γ-Glutamyl transpeptidase-positive foci induction, female Sprague-Dawley rat liver *in vivo*	–		100 po × 6 (weekly)	Martelli *et al*. (1996b)
Unscheduled DNA synthesis, male and female human hepatocytes *in vitro*	+	NT	0.77	Martelli *et al*. (1996a)
Chromosomal aberrations, human lymphocytes *in vitro*	–	NT	10	Stenchever *et al*. (1969)
Micronucleus formation, female rat liver *in vivo*	–		100 po × 1	Martelli *et al*. (1996b)
Binding (covalent) to DNA, female rat hepatocytes *in vitro*	+	NT	1.2	Topinka *et al*. (1995)
Binding (covalent) to DNA, male rat hepatocytes *in vitro*	(+)	NT	1.2	Topinka *et al*. (1995)
Binding (covalent) to DNA, female rat liver *in vivo*	(+)		10 po × 1	Topinka *et al*. (1995)
Norethynodrel				
Salmonella typhimurium TA100, TA1535, TA1537, TA1538, TA98, reverse mutation	–	–	1000 µg/plate	Lang & Redmann (1979)
Salmonella typhimurium TA100, TA1535, TA1537, TA1538, TA98, reverse mutation	–	–	250 µg/plate	Rao *et al*. (1983)
Salmonella typhimurium TA100, TA1535, TA1537, TA1538, TA98, reverse mutation	–	–	2000 µg/plate[d]	Lang & Reimann (1993)
Inhibition of metabolic cooperation, Chinese hamster V79 cells *in vitro*	(+)	NT	0.75	Yager (1983)
Norethynodrel + 2-acetylaminofluorene (3 µg)				
Salmonella typhimurium TA100, TA1535, TA98, reverse mutation	NT	–	150 µg/plate	Rao *et al*. (1983)

Table 46 (contd)

Test system	Result[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Levonorgestrel				
Salmonella typhimurium TA100,TA1535, TA1537, TA1538, TA98, reverse mutation	–	–	2500 µg/plate	Lang & Redmann (1979)
Salmonella typhimurium TA100,TA1535, TA1537, TA1538, TA98, reverse mutation	–	–	1000 µg/plate	Dayan *et al*. (1980)
Salmonella typhimurium TA100,TA1535, TA1537, TA1538, TA98, reverse mutation	–	–	500 µg/plate	Lang & Reimann (1993)
Drosophila melanogaster, sex-linked recessive lethal mutations	–		3120	Parádi (1981)
Drosophila melanogaster, sex-linked recessive lethal mutations	(+)		5000	Aguiar & Tordecilla (1984)

[a] +, positive; (+), weak positive; –, negative; NT, not tested
[b] LED, lowest effective dose; HID, highest ineffective dose; in-vitro tests, µg/mL; in-vivo tests, mg/kg bw per day; po, oral; ip, intraperitoneal
[c] Total mixture
[d] Toxicity was observed at higher dose(s)

4.3.10 *Norethynodrel* (Table 46)

Gene mutations were not induced in *S. typhimurium* by norethynodrel, and it did not enhance the mutagenicity of a sub-threshold mutagenic dose of 2-acetylaminofluorene.

4.3.11 *Levonorgestrel* (Table 46)

Gene mutations were not induced in *S. typhimurium* by levonorgestrel. Ethanolic extracts of combinations of levonorgestrel and ethinyloestradiol induced cell transformation in baby rat kidney cells infected with HPV-16 and carrying the Ha-*ras*-1 oncogene (see Table 42).

4.4 Reproductive and prenatal effects

The literature up to 1979 on the developmental effects of sex hormones was reviewed in Volume 21 of the *IARC Monographs* (IARC, 1979). It has been shown in both humans and experimental animals that sex hormones can interfere with normal genital development. The effects observed with synthetic sex hormones are variable, and oestrogenic, androgenic and progestogenic effects may frequently be observed with one chemical, depending on the target tissues and the background levels of natural hormones acting at specific times. The effects on embryofetal development also depend on the relative importance of numerous conditioning factors and are not always easy to predict; however, masculinization of female fetuses and feminization of male fetuses are observed. Effects are found in many organ systems, and genital development, central nervous system development and sexual differentiation may be affected. The timing of exposure relative to embryofetal and postnatal development is critical in determining the type and site of the defect produced.

The literature on the effects of exposure to sex hormones during pregnancy on induction of other types of congenital malformation is much more controversial. Early case reports and epidemiological studies suggested that a wide variety of defects, affecting most organ systems, could be produced. Syndromes such as the VACTERL syndrome were reported, which involves malformations of one or more of the vertebral, anal, cardiac, tracheal, oesophageal, renal and limb systems. Numerous other studies failed to support the suggestion that these defects were related to hormonal treatment.

Three categories of exposure in pregnancy were considered. Accidental exposure to oral contraceptives comprised the major group, with the least convincing evidence for a connection with birth defects. The evidence related to use of hormonal pregnancy tests was a little stronger but still unsubstantiated; the use of such tests was discontinued many years ago. The third category is use of hormones to treat women with pregnancy problems, such as intermittent bleeding, repeated or threatened abortion and luteal failure. In those cases in which the pregnancy is maintained but the fetus has malformations, it is difficult to decide whether the cause was the hormonal treatment or the underlying disease.

Since the last IARC monograph on this subject (IARC, 1979), many papers have been published on the topic of exposure to hormones during pregnancy, and some have been reviewed. Schardein (1980b) reviewed the literature up to that time on the induction

of genital and non-genital defects. He concluded that the commonest association for genital defects was masculinization of females, generally seen as clitoral hypertrophy, with exposure at around week 8–10 of pregnancy; the prevalence was low and the risk was estimated to be about 1% of exposed infants. The evidence for feminization of males was reported to be less convincing. Hypospadia has been the commonest defect reported, but recent analysis of more than 2000 cases of hypospadia has shown no association with maternal use of oral contraceptives (Källén *et al*., 1991). A meta-analysis of pregnancy outcomes after exposure to sex hormones during the first trimester showed no excess of genital malformations (Raman-Wilms *et al.*, 1995).

The evidence accumulated since 1979 on the involvement of exposure to sex hormones in other non-genital malformations has been largely in favour of no association. In a short paper, Brent (1994) reviewed some of the reasons why false associations between congenital heart malformations and hormones may have been concluded in the past. These include the grouping of many different types of congenital heart disorder with different causes, inadequate knowledge about the critical times of exposure for specific defects, failure to differentiate between the actions of oestrogens and progestogens, and inclusion in some studies of syndromes with a known high incidence of heart defects. The paper lists 20 reviews on the subject of exposure to hormones and non-genital congenital malformations, none of which found a causal association. It can be concluded that most epidemiological studies do not indicate that progestogens are teratogenic for the cardiovascular system; secular trend data do not support an association between exposure to sex hormones and cardiovascular disease; a very large number of experimental studies show no relationship between exposure to sex hormones and cardiac malformations; there are no sex hormone receptors in developing cardiac tissue; and no consistent syndrome of non-genital defects has been reported. In 1988, as a result of a meeting to review the evidence, the United States Food and Drug Administration removed the warning label on oral contraceptives which had previously stated that exposure during pregnancy could cause cardiac and limb defects (Brent, 1989).

Useful reviews of the data on congenital malformations have been published (Wilson & Brent, 1981; Polednak, 1985; Simpson, 1985; Bracken, 1990; Simpson & Phillips, 1990). Several case–control studies have been conducted that show little or usually no evidence of an association between birth defects and hormonal exposure (Lammer & Cordero, 1986; Hill *et al*., 1988; Ananijevic-Pandey *et al*., 1992; Pradat, 1992; Martínez-Frías *et al*., 1998). In a large cohort study in the United States (Harlap *et al*., 1985a), no increase in the incidence of malformations was found in relation to the use of oral or other methods of contraception. In a study of women in Thailand (Pardthaisong *et al*., 1988), no increase in the incidence of defects of the heart, central nervous system or limbs was found in the offspring of women using oral or injectable contraceptives, but an increased incidence of polysyndactyly and chromosomal anomalies was observed in women who had previously used medroxyprogesterone acetate (Depo Provera). The small numbers of affected children, the long interval between injection of medroxyprogesterone and the conception of the affected offspring and the unrelated nature of the

effects led the authors to conclude that a causal relationship between treatment and effect was unlikely. Overall, the prevalence of major malformations was significantly lower in the oral contraceptive users than in the non-users.

A study of oral contraceptive use in 730 mothers of children with Down syndrome and 1035 mothers of children with other malformations (Lejeune & Prieur, 1979) showed that more of the mothers aged 30–38 years of children with Down syndrome had ceased contraceptive use within the six months prior to conception than in the other group. Three other studies, two of them prospective studies in which data on contraceptive use was obtained before the outcome of the pregnancy was known (Ericson *et al*., 1983; Källén, 1989), and a smaller case–control study of contraceptive use in the mothers of Down cases and in mothers of normal children (Janerich *et al.*, 1976), found no association between use of oral contraceptives and the subsequent birth of a child with Down syndrome.

A study of chromosomal abnormalities in 33 551 births and abortions after 20 weeks was reported by Harlap *et al*. (1985b). No increased risk was found for women who used oral contraceptives prior to conception or who were still using contraceptives when they became pregnant.

4.4.1 *Ethinyloestradiol*

(*a*) *Humans*

No data were available to the Working Group.

(*b*) *Experimental systems*

A study was carried out with the inbred mouse strain 129SV-S1 C P, which is heterozygous for the *S1* gene shown to affect the development of primordial germ cells, and which has a 7% spontaneous incidence of testicular teratoma. Pregnant mice were injected subcutaneously with 0.02 or 0.2 mg/kg bw ethinyloestradiol in corn oil on days 11 and 12 of gestation (the day a vaginal plug was observed was considered to be day 0), which had been shown previously to be the critical period for induction of teratoma. The male pups were killed at 15 days of age and the testes examined for teratomas. A dose-related increase in the incidence of cryptorchid testes was found, with 4/107 in controls, 10/109 at the low dose and 23/115 at the high dose ($p < 0.0001$ for trend). A small increase was observed in the incidence of teratoma which was neither dose-related nor significant (odds ratio, 2.4; 95% CI, 0.7–9.1) for the pooled data. The authors suggested that different mechanisms are involved in the etiology of cryptorchidism and teratoma, although both may be induced by oestrogen stimulation (Walker *et al.*, 1990).

Oral administration of 0, 0.02, 0.2 or 2.0 mg/kg bw ethinyloestradiol in olive oil to pregnant Jcl:ICR mice (8–15 litters per group) on days 11–17 of gestation (the presence of a vaginal plug being considered day 0) resulted in a dose-dependent increase in the incidences of ovotestis and cryptorchidism in males, with persistent Müllerian and Wolffian ducts, when fetuses were examined on day 18 of gestation. Leydig-cell proliferation was also seen at the two higher doses with alterations in cellular morphology

suggestive of preneoplastic changes. Female fetuses showed ovarian hypoplasia, with decreased numbers of primordial follicles and increased follicular degeneration (Yasuda *et al.*, 1985, 1986).

In a later study (Yasuda *et al*., 1988), pregnant ICR mice were given a daily dose of 0.02 or 0.2 mg/kg bw ethinyloestradiol in olive oil orally on days 11–17 of gestation. Dams at the low dose were allowed to deliver, and their male offspring were reared to maturity (20–22 months). The animals given the high dose had no live offspring. At day 18, male fetuses were recovered from mice at each dose, and the concentrations of testosterone and oestradiol were measured in testes. Ethinyloestradiol treatment significantly ($p < 0.001$) reduced the concentrations of testosterone in the testes of 18-day-old fetuses, from a mean of 5.21 ± 0.13 pg/testis in the controls ($n = 32$ testes) to 0.89 ± 0.11 pg/testis ($n = 30$) at the low dose and 0.40 ± 0.13 pg/testis ($n = 52$) at the high dose. Treatment at the high dose also reduced the oestradiol levels in the testis from 78.80 ± 0.49 pg/testis in controls to 42.25 ± 1.56 pg/testis ($p < 0.05$). The testosterone:oestradiol ratios were reduced from 1:15 in controls, to 1:77 at the low dose and 1:106 at the high dose. At 20–22 months, the offspring from the low-dose group were killed, and the testes and epididymides were removed, examined histologically and analysed for testosterone and oestradiol. There were significant ($p < 0.05$) increases in the frequency of testicular atrophy, Leydig-cell hyperplasia and absence of epididymal sperm in the treated compared with the control mice. The testosterone concentration was significantly decreased, from 84 to 28 ng/testis ($p < 0.01$), and the oestradiol level was increased, from 356 to 564 ng/testis ($p < 0.05$). The authors suggested that the dramatic fall in the testosterone:oestradiol ratio in the fetal testis results from greatly increased conversion of testosterone to oestradiol by the Leydig cells, so that insufficient amounts of testosterone are available for regulation of pro-spermatogenesis in the developing testis and spermatogenesis, eventually resulting in sterility.

4.4.2 *Mestranol*

(*a*) *Humans*

No data were available to the Working Group.

(*b*) *Experimental systems*

Daily oral administration of 0.05 or 0.2 mg/kg bw mestranol to female NMRI mice and $AFAF_1$ hybrid mice, on days 4–8 after mating, inhibited implantation and increased the number of resorptions. The fetuses of NMRI mice had accessory ribs. Treatment on days 7–11 with doses of 0.1–0.2 mg/kg bw induced abortions but had no teratogenic effects (Heinecke & Klaus, 1975).

In rats, subcutaneous injection of 0.002–0.02 mg/kg bw mestranol five days before and 30 days after mating prevented implantation in a dose-dependent manner. Subcutaneous injection of 0.02 mg/kg bw or oral administration of 0.1 mg/kg bw on days 2–4 of gestation terminated pregnancy (Saunders & Elton, 1967).

Charles River rats received daily oral doses of 0.05–0.2 mg/kg bw Enovid (2.5 mg norethynodrel, 0.1 mg mestranol) or 0.01–0.1 mg/kg bw mestranol throughout gestation

and for 21 days after parturition. The highest dose of mestranol terminated a significant percentage of pregnancies. No genital defects were observed in surviving male offspring, but female offspring showed enlarged urethral papillae and prematurely opened vaginas, even at lower doses of mestranol. The fertility of female offspring of rats treated with 0.1 mg Enovid was impaired by 55%. Higher doses of Enovid or 0.02 mg mestranol induced complete sterility in female offspring; examination of the ovaries showed no corpora lutea and follicles of reduced size (Saunders, 1967).

Sixty female Wistar rats were given a daily dose of 1 mg/kg bw Enidrel (0.075 mg mestranol, 9.2 mg norethynodrel) intragastrically for two months, at which time they were mated. In 30 animals in which treatment was continued, complete fetal resorption occurred rapidly; however, after two weeks without treatment, the fertility rates and litter sizes were normal. In the 30 animals in which treatment was discontinued, the fertility and pre- and post-natal development of the offspring were also normal. No teratogenic effects were observed (Tuchmann-Duplessis & Mercier-Parot, 1972).

In rabbits, pregnancy was terminated by daily oral doses of > 0.02 mg/kg bw mestranol on days 0–28 or 0.05 mg/kg bw [lower doses not tested] on days 10–28 of gestation and by daily subcutaneous doses of 0.005 mg/kg bw on days 0–28 or > 0.002 mg/kg bw on days 10–28. Doses that did not terminate pregnancy had no effects on litter size or the weights of the offspring (Saunders & Elton, 1967).

In female Syrian golden hamsters that received a contraceptive steroid containing 18.7 μg mestranol and 0.6 mg lynoestrenol [route unspecified] daily for 4.5–8 months, fertility was found to be normal; no effects were seen on sexual behaviour or on the fecundity of the offspring of the following two generations (Cottinet *et al*., 1974).

When adult beagle bitches received 5 mg/kg bw mestranol orally on day 6 or 21 of gestation, the embryonic losses, based on corpora lutea counts, were 95.5% with early treatment and 67.3% with late mestranol treatment in comparison with 34.5% in controls. The surviving offspring appeared normal (Kennelly, 1969).

4.4.3 *Chlormadinone acetate*

(*a*) *Humans*

No increase in the incidence of malformations was reported in 305 infants whose mothers had been exposed to chlormadinone and oestrogens during pregnancy (Goldzieher *et al*., 1968; Lepage & Gueguen, 1968; Larsson-Cohn, 1970).

(*b*) *Experimental systems*

Groups of 8–12 male Sprague-Dawley Crl:CD(SD)Br rats were castrated and injected immediately thereafter twice daily for 14 days with one of a number of synthetic progestogens, including chlormadinone acetate, used in the treatment of prostate cancer. Controls received the vehicle, 1% gelatine in 0.9% saline. Dihydrotestosterone was injected at a dose of 150 μg twice daily for 14 days as a positive control. All animals were killed on the morning after the last day of treatment, and the ventral prostate and adrenals were removed and weighed; furthermore, the prostatic content of ornithine decarboxylase was

measured, as it is considered to be a highly specific, sensitive marker of androgenic activity in the prostate. Dihydrotestosterone increased the ventral prostate weight to 43% above that of castrated controls. Chlormadinone acetate was less potent than dihydrotestosterone but caused significant increases in prostate weight, by about 22% at 3 mg and 36% at 10 mg per injection. Whereas dihydrotestosterone caused a 14-fold increase in ornithine decarboxylase activity in the prostate, chlormadinone acetate caused a 5.3-fold increase at 3 mg and an 11.8-fold increase at 10 mg. Chlormadinone acetate thus has weak but significant androgenic activity in the rat ventral prostate (Labrie *et al.*, 1987).

Pregnant Wistar rats were given 1, 5 or 10 mg chlormadinone acetate orally once a day for four days on days 17–20 of pregnancy, and the fetuses were removed on day 21. After fixation, histological sections of the pelvic region were examined and the urovaginal septum length measured. Masculinization of female fetuses was not observed, in the absence of change in the development of the urogenital septum (Kawashima *et al.*, 1977).

Chlormadinone acetate given orally at doses of 1–50 mg/kg bw on days 8–15 of pregnancy to Japanese ddS and CF1 mice caused a significant increase in the incidence of cleft palate. A dose of 10 mg/kg bw, but not of 1 or 3 mg/kg bw, given orally on days 8–20 of gestation to Japanese albino rabbits increased the incidence of cleft palate, abdominal wall defects and wrist contractures (Takano *et al.*, 1966).

4.4.4 *Cyproterone acetate*

(*a*) *Humans*

Two men treated for prostatic carcinoma with high oral doses of cyproterone acetate (2×100 mg per day for seven months) had widespread testicular damage, with disappearance of Sertoli cells and spermatogonia and involution of Leydig cells (Re *et al.*, 1979). When cyproterone acetate was given at doses of 5–10 mg per day as a contraceptive in several other studies, decreased sperm concentration and motility and increased abnormal morphology, with—except in one study—decreased power to penetrate the mucus, were observed. Variable effects on plasma gonadotrophins and testosterone levels have been reported (Føgh *et al.*, 1979; Roy & Chatterjee, 1979; Moltz *et al.*, 1980; Wang & Yeung, 1980). Doses of 50–100 mg cyproterone acetate per day combined with testosterone induced azoospermia and decreased testis size in each of 10 subjects. All of the effects were reversible (Meriggiola *et al.*, 1996).

In women, ovulation is inhibited by 2 mg per day cyproterone acetate when given in combination with 35 µg ethinyloestradiol (Spona *et al.*, 1986). No controlled studies on developmental effects are available.

(*b*) *Experimental systems*

Cyproterone acetate has been reported to have both androgenic and anti-androgenic activity in experimental animals (see also section 4.2.5).

Groups of 8–12 male Sprague-Dawley Crl:CD(SD)Br rats were castrated and then injected twice daily for 14 days with one of a number of synthetic progestogens,

including cyproterone acetate. Treatment was begun one day after castration. Controls were injected with the vehicle, 1% gelatine in 0.9% saline. Dihydrotestosterone was injected twice daily at a dose of 125 μg for 14 days as a positive control. All animals were killed on the morning after the last day of treatment, and the ventral prostate and adrenals were removed and weighed. Dihydrotestosterone increased the ventral prostate weight to approximately five time that of castrated controls. Cyproterone acetate was less potent than dihydrotestosterone but caused a significant increase in prostate weight, by 60% at a dose of 5 mg per injection twice daily. Cyproterone acetate thus has weak but significant androgenic activity in the rat ventral prostate (Poyet & Labrie, 1985).

Anti-androgenic effects have also been reported. Groups of 10 albino Wistar mice were treated subcutaneously with vehicle alone or with 1 mg per animal per day of cyproterone acetate for seven days. The animals were killed on the eighth day and the testes removed for histological and morphometric examination. Treatment caused marked decreases in the volume, surface area and length of the seminiferous tubules, and it inhibited spermatogenesis (Umapathy & Rai, 1982).

The anti-androgenic activity of cyproterone and cyproterone acetate has been shown in mice (Umapathy & Rai, 1982; Homady *et al.*, 1986), rats (El Etreby *et al.* 1987), guinea-pigs (Tam *et al*., 1985), ferrets (Kästner & Apfelbach, 1987), goats (Panda & Jindal, 1982; Kumar & Panda, 1983) and monkeys (Lohiya *et al*., 1987; Kaur *et al*., 1990, 1992). The effects observed include decreased sexual behaviour and inter-male aggression, reduced weights of testis and inhibition of spermatogenesis. Fertility can be reduced by low doses of cyproterone acetate even in the absence of reduced spermatogenesis (Rastogi *et al*., 1980), which may be due to an effect on epididymal processing of sperm. In addition to reduced secretion of testosterone and luteinizing hormone (Clos *et al*., 1988), there is also evidence that translocation of the testosterone receptor to the nucleus may be affected (Brinkmann *et al*., 1983).

In rodents, cyproterone acetate has oestrogenic properties, increasing uterine weight and causing vaginal cornification in ovariectomized rats (Arya *et al.*, 1979). When the compound was administered to pregnant rats, feminization of male fetuses, including development of a vagina, has been reported (Neumann *et al.*, 1966; Forsberg & Jacobsohn, 1969).

Treatment of NMRI mice with doses of 5–900 mg/kg bw cyproterone acetate subcutaneously on day 2 of gestation (the day a vaginal plug was observed was considered to be day 0) or with 30 mg/kg bw on single days of pregnancy from day 1 to 12, resulted in a clear dose- and time-related increase in the incidences of cleft palate and of urinary tract and respiratory tract malformations, with up to 64% of fetuses affected after the single 900-mg/kg bw dose (Eibs *et al.*, 1982). Administration in late pregnancy or in the neonatal period can produce permanent changes in neuroendocrine and sexual function of rats. Groups of 15 male and 16 female offspring of rats treated subcutaneously with 1 mg cyproterone acetate on days 15–20 of gestation [strain and number of pregnant animals not specified] were studied when two to three months of age. The weight of the brain was reduced in animals of each sex and the weight of the testis in males. Cell

density in the ventromedial nucleus of the hypothalamus was increased in males in comparison with females. The prolactin concentration in the pituitary was increased in animals of each sex (Rossi *et al.*, 1991). Groups of newborn male Swiss CD1 mice were injected with cyproterone acetate on days 1–10 (200 μg/day), 11–20 (400 μg), 21–30 (800 μg) or 31–40 (1 mg) of age. In all groups, there was an immediate reduction in the weights of the testis, epididymis, vas deferens, preputial gland and seminal vesicles relative to body weight. This reduction in the weight of the accessory sex organs was permanent in animals injected up to day 20 of age but was reversible in animals treated after day 20. In mice injected on days 1–10 of age, marked, permanent infertility was observed when they became adults, but spermatogenesis, the androgen concentration in plasma and sexual behaviour were not affected. The infertility appeared to be due to failure of sperm in the epididymis to mature (Jean-Faucher *et al.*, 1985).

4.4.5 *Levonorgestrel* (see also the monograph on 'Hormonal contraceptives, progestogens only', section 4.4.2)

(*a*) *Humans*

No relevant data were available to the Working Group.

(*b*) *Experimental systems*

Groups of six pregnant rats [strain not specified] were ovariectomized on day 8 of gestation and treated subcutaneously with doses of 0.01–0.3 mg levonorgestrel daily on days 8–21 of gestation; at the same time they received an injection of 1.0 μg oestrone. The rats were killed on day 22 to measure maintenance of pregnancy; satisfactory maintenance was achieved with 0.1 and 0.3 mg levonorgestrel. Immature castrated male rats [age not specified] were treated subcutaneously daily for 13 days with doses of 0.1–3 mg levonorgestrel and were killed the day after the last treatment; the weights of the prostate and seminal vesicles were measured. Levonorgestrel showed androgenic activity, as judged from the increased weight of both tissues (Kuhnz & Beier, 1994).

Groups of 10–12 Prob:WNZ New Zealand white rabbits were mated with Prob:KAL Californian rabbits, producing hybrid fetuses; the day of mating was called day 1. The animals were treated with 0.5 mg/kg bw levonorgestrel in sesame oil by gavage on days 5–25 of gestation and were killed on day 21. [The Working Group concluded that the animals must have been killed after day 25, but the fetal body weights were very low for full-term offspring.] The fetuses were examined macroscopically, and half of them were sliced for visceral examination and the other half examined for skeletal and cartilage malformations. No adverse effect of treatment on pregnancy rate, number of implantations, number of resorptions or number of live or dead fetuses was observed. The female fetal body weight at term was slightly but significantly reduced (15.2 ± 0.61 g versus 17.4 ± 0.74 g in the sesame oil control group). No malformations were observed (Heinecke & Kohler, 1983).

4.4.6 *Lynoestrenol*

(*a*) *Humans*

No data were available to the Working Group.

(*b*) *Experimental systems*

Groups of 14–20 inseminated belted Dutch rabbits were given lynoestrenol at a dose of 0, 0.1, 0.5 or 2.5 mg/kg bw orally on days 6–18 of gestation (the day of insemination being considered day 0). The does were killed on day 29 for examination of the fetuses. Increased post-implantation loss was observed at all doses: 1.3% in controls, 13.2% at the low dose, 17.1% at the intermediate dose and 90.9% at the high dose. A wide range of malformations of the brain and eye was observed in all groups, affecting 31–50% of fetuses and only 12% of controls. Cardiovascular malformations were observed in 2, 5 and 20% of fetuses, respectively, at the three doses. No masculinization of female fetuses was observed. In a second experiment with groups of five to six belted Dutch rabbits, lynoestrenol was administered at a dose of 0, 0.1 or 0.5 mg/kg bw on days 6–18 of gestation. The does were allowed to deliver naturally and raise their pups to four weeks of age. At the higher dose, the litter size was decreased and there was clear evidence of central nervous system abnormalities in surviving pups, with ataxia, disorientation, posterior paralysis and rotation of one or both hindlimbs. Anophthalmia and microphthalmia were seen in 3/10 pups at the high dose. Histological examination of the central nervous system revealed pathological changes in the ventral horns of the spinal cord, with a marked reduction in the number of neurons (Sannes *et al.*, 1983).

Pregnant Wistar rats were given 1 or 5 mg lynoestrenol orally once a day for four days on days 17–20 of gestation, and the fetuses were removed on day 21. After fixation, histological sections of the pelvic region were examined and the urovaginal septum length measured. Masculinization of female fetuses was seen at 5 mg, as evidenced by decreased development of the urogenital system (Kawashima *et al.*, 1977).

4.4.7 *Megestrol acetate*

(*a*) *Humans*

No data were available to the Working Group.

(*b*) *Experimental systems*

Megestrol acetate has some androgenic activity in rats (Poyet & Labrie, 1985; Labrie *et al*., 1987). In the study of Labrie *et al.* (1987), described in section 4.4.3, the positive control dihydrotestosterone increased the ventral prostate weight to 43% above that of castrated controls. Megestrol acetate was less potent, but caused significant increases in prostate weight, by about 35% at 3 mg and 59% at 10 mg per injection. Whereas dihydrotestosterone caused a 14-fold increase in the activity of orthinine decarboxylase in the prostate, megestrol acetate caused an 11-fold increase at 3 mg and a 13-fold increase at 10 mg. Megestrol acetate thus has weak but significant androgenic activity in the rat ventral prostate.

In the study of Kawashima *et al*. (1977) described in section 4.4.3, megestrol acetate at a dose of 5 mg induced masculinization of female fetuses.

4.4.8 *Norethisterone*

(*a*) *Humans*

No data were available to the Working Group.

(*b*) *Experimental systems*

Ten groups of three timed-mated pregnant rhesus monkeys (*Macaca mulatta*) were treated with Norlestrin (norethisterone acetate, 2.5 mg and ethinyloestradiol, 0.05 mg per tablet) orally at a dose of 5, 10, 25 or 50 mg per monkey per day (on the basis of the norethisterone acetate content). [These doses are equivalent to 0.83, 1.67, 4.17 and 8.33 mg/kg bw daily on the basis of the information in the paper that the doses are equivalent to 20, 40, 100 and 200 times the human contraceptive dose.] The animals at the three lower doses were treated daily during early (days 21–35) or late (days 33–46) organogenesis or throughout (days 21–46) organogenesis and were allowed to deliver at term (165 days' gestation). Animals at 50 mg/day were dosed on days 21–35 only and delivered by caesarean section on day 50 of gestation for serial sectioning and histological examination of the fetuses. Of 26 animals that were allowed to deliver at term, 16 delivered morphologically normal infants (nine male, seven female), eight aborted, and two had stillbirths, a rate of 7.4%, which was not different from that of controls. The overall pre-natal mortality rate was higher in the treated animals (10/26, 38.5%) than in the control colony (55/262, 21%). Two of nine animals at each of the 5- and 10-mg doses aborted, in comparison with 4/9 at 25 mg/day. Among those treated on days 21–35 or 21–46 of gestation, six (37.5%) aborted, in comparison with 2/9 (22.2%) of those treated later in organogenesis, on days 33–46. Only three aborted embryos were recovered, and all three were much smaller than expected for their gestational age; however, they were too severely autolysed for further examination. No morphological or histological abnormalities were detected in fetuses recovered on day 50 of gestation from females at 50 mg. The infants were followed up for a maximum of 2.5 years, and all three animals that died and the five that were sacrificed were necropsied and examined histopathologically. No malformations or significant lesions were found. Detailed physical examination of the live infants showed no morphological changes, and the body weights and other measures were no different from those of controls. The serum oestrogen and progesterone concentrations of females treated with 25 mg on days 21–35 of gestation were measured daily on days 26–44 by immunoassay. The oestrogen concentrations were significantly lower ($p < 0.05$) than those of controls, but the progesterone levels were similar. As it has been shown in other studies that monkeys can be ovariectomized on day 23 of gestation without fetal loss, although the plasma oestrogen concentration falls almost to zero, the authors suggested that the reduction in oestrogen concentration was not the cause of the observed pre-natal deaths. They proposed that Norlestrin has a direct embryolethal effect. The normal progesterone concentrations indicate that

placental synthesis of progesterone is unaffected. The authors also pointed out that as the periods of treatment in this study did not extend into the early fetal period after day 46, when external genital development occurs in the rhesus monkey, genital malformations would not be expected to occur (Prahalada & Hendrickx, 1983).

Eight of the offspring in the study described above from females dosed with 5, 10 or 25 mg Norlestrin were subjected to limited behavioural examination up to 11 months of age. No serious deficiencies in the regulation of activity, motor maturity, manual dexterity or discrimination learning were observed at three to five months of age. Age-appropriate sex-differentiated behaviour was seen at five and 11 months of age. The authors noted, however, that Norlestrin was not given during the period of sexual differentiation of the brain in rhesus monkeys (Golub *et al.*, 1983).

In the study of Kawashima *et al.* (1977), described in section 4.4.3, norethisterone caused masculinization of female fetuses at doses of 5 or 10 mg but not at 1 mg.

4.4.9 *Norethynodrel*

(*a*) *Humans*

No data were available to the Working Group.

(*b*) *Experimental systems*

Mice given 10 mg/kg bw norethynodrel (with 2% mestranol) orally, daily on days 8–15 of gestation, had a very high rate of embryonic death (98.9%), but not when the dose was given on days 14–17 of gestation (Takano *et al*., 1966).

In mice given 0.2–2.4 mg/kg bw norethynodrel or its 3-hydroxy metabolite as an oral or parenteral dose, either singly or on three consecutive days between days 6 and 16 of gestation, congenital anomalies were observed in near-term fetuses. A single dose of 1.2 mg/kg bw norethynodrel or its metabolite given between days 8 and 16 of gestation produced congenital abnormalities (retarded development, hydrocephalus, club-foot and minor skeletal anomalies) in 10–30% of offspring (Andrew *et al*., 1972).

Mice that received a single subcutaneous injection of 0.1 mg/kg bw norethynodrel in combination with 1.5 µg/kg mestranol (Enovid) on day 7, 10, 12, 15 or 17 of gestation had normal fetuses, with no external or internal genital anomalies; however, treatment on day 10 of gestation led to a significant decrease in aggressive behaviour of male offspring later in life (Abbatiello & Scudder, 1970).

Oral administration of norethynodrel or its metabolites, 17α-ethynyl-oestr-5(10)-ene-3α,17β-diol and 17α-ethynyl-oestr-5(10)-ene-3β,17β-diol, at a daily dose of 0.15, 0.3 or 0.6 mg/kg bw on days 8–10 or 11–13 of gestation resulted in increased numbers of resorptions and intrauterine deaths on days 11–13. The teratogenic effects included exencephaly after treatment during days 8–10 and hydrocephalus and partial cryptorchidism after treatment on days 11–13. The most effective agent was 17α-ethynyl-oestr-5(10)-ene-3β,17β-diol (Gidley *et al.*, 1970).

Subcutaneous administration of 0.5 or 1 mg/kg bw norethynodrel to pregnant rats on days 2–4 of gestation terminated a significant number of pregnancies (Saunders, 1965).

Subcutaneous administration of 0.083–2.5 mg/kg bw per day norethynodrel to rats on days 10–17 of gestation induced 100% fetal resorptions, whereas a dose of 0.0083 mg/kg bw per day induced 42% resorptions; no virilizing effect was observed in females, but in males the weight of the testes was significantly lowered and the descent of testes was delayed in 35.5% of animals (Roy & Kar, 1967).

In the study of Tuchmann-Duplessis and Mercier-Parot (1972), described in section 4.4.2, complete fetal resorption occurred rapidly in 30 animals in which treatment with Enidrel (mestranol/norethynodrel) was continued for the first 15 days of gestation; however, after two weeks without treatment, the fertility rates and litter sizes were normal. In the 30 animals in which treatment was discontinued, the fertility and pre- and post-natal development of the offspring were normal. No teratogenic effects were observed.

Subcutaneous injection of 1 mg norethynodrel (Enovid) to guinea-pigs daily on days 18–60 of gestation prevented pregnancy (Foote *et al*., 1968).

4.4.10 *Norgestimate*

(*a*) *Humans*

No data were available to the Working Group.

(*b*) *Experimental systems*

In the study of Kuhnz and Beier (1994), described in section 4.4.5, groups of rats were given doses of 0.03–1 mg/day norgestimate. Satisfactory maintenance of pregnancy was achieved with 0.3 and 1 mg. In immature male rats treated with 1–30 mg norgestimate, the weights of the prostate and seminal vesicles were increased, indicating androgenic activity, but norgestimate was less active than levonorgestrel. The blood concentrations of levonorgestrel were measured in animals treated with levonorgestrel or norgestimate, as levonorgestrel is the major metabolite of norgestimate. Both the pregnancy maintenance and the androgenic activity could be accounted for by the concentrations of levonorgestrel produced as a metabolite.

4.4.11 *Norgestrel*

(*a*) *Humans*

As the active isomer of norgestrel is levonorgestrel, the reader is referred to section 4.4.5 of this monograph.

(*b*) *Experimental systems*

Mature female B3C6F_1 mice were superovulated with serum gonadotropin from pregnant mares and 48 h later by human chorionic gonadotrophin; they were then mated overnight with males known to be fertile. The females were killed, and the fertilized pre-embryos were collected from the fallopian tubes 24 h (one-cell stage) or 48 h (2–4-cell stage) after the injection of human chorionic gonadotrophin. The pre-embryos were then cultured for up to 72 h in the absence or presence of 4 ng/mL (dl)-norgestrel, which is the peak plasma concentration of levonorgestrel found in women who use norgestrel as

a contraceptive. [It is unclear whether this dose refers to (dl)- or levonorgestrel.] After 24, 48 or 72 h of culture, the pre-embryos were examined microscopically, and the number of cells counted up to the morula and late blastocyst stages. A similar experiment was carried out in which one-cell pre-embryos were harvested 24 h after injection of human chorionic gonadotrophin and exposed in culture to norgestrel at a concentration of 8, 80 or 800 ng/mL for up to 72 h. In neither study was any difference found in the number of pre-embryos at various cell stages or in the number of degenerating or abnormal pre-embryos (Logan *et al.*, 1989).