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

## 4.1 Absorption, distribution, metabolism and excretion

The disposition of oestradiol, oestrone and oestriol is considered together, because there is interconversion between oestradiol and oestrone *in vivo* in both humans and other mammals, and the latter is converted to oestriol (Figure 4).

Various preparations of oestradiol, such as crystalline oestradiol, micronized oestradiol and esterified oestradiol (e.g. oestradiol valerate, oestradiol 3-benzoate, oestradiol dipropionate), are used for post-menopausal hormonal therapy. The absorption of these oestradiol preparations differs, while the route of exposure remains the same. For example, crystalline oestradiol applied dermally in a cream diffuses more readily through the skin to the systemic circulation than esterified oestradiol, because oestradiol is more lipophilic than its ester derivative. Similarly, micronized oestradiol is absorbed more rapidly than crystalline oestradiol because of its small particle size. The absorption of these oestradiol preparations also depends on the dose administered and the route of administration.

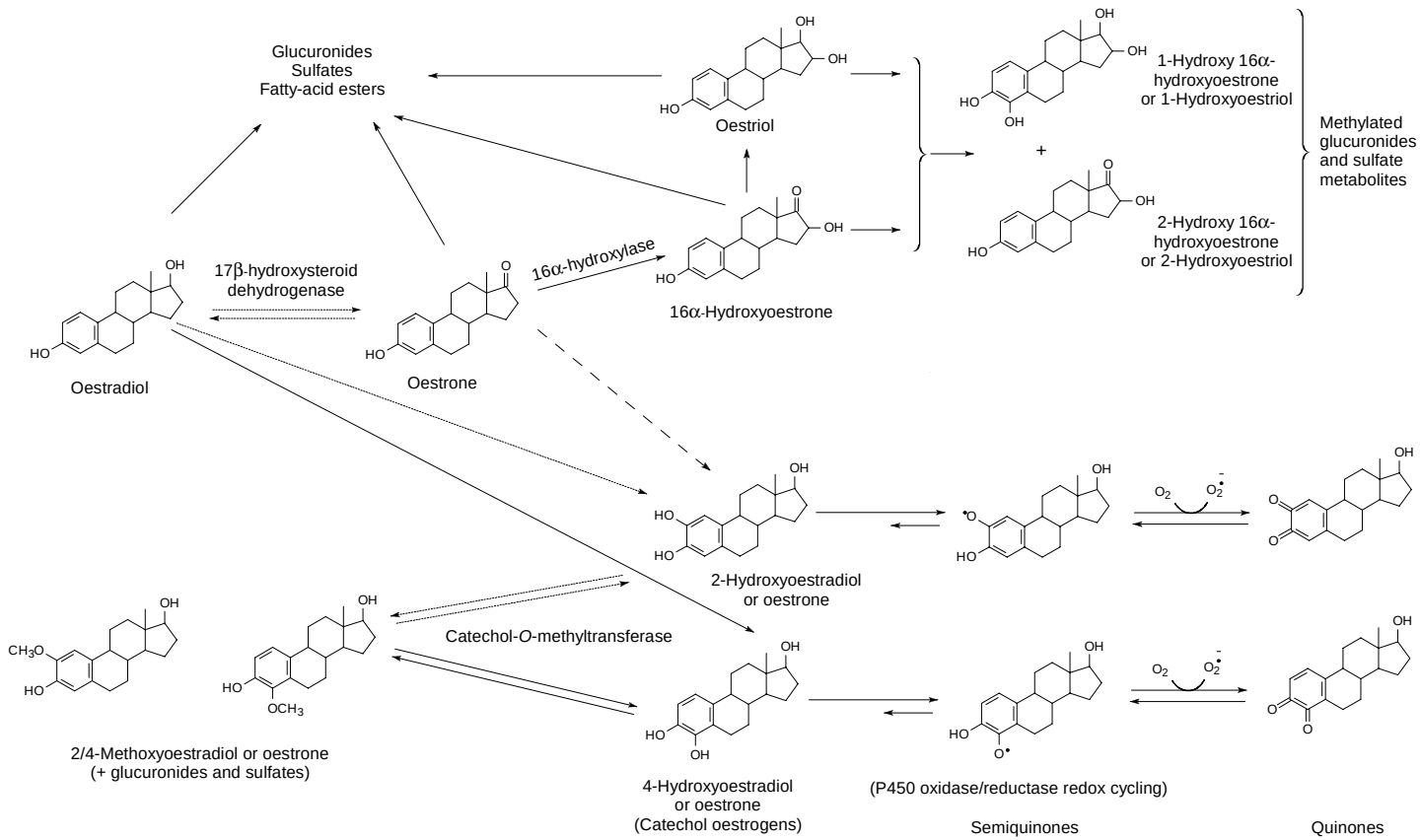
The pharmacokinetics of conjugated equine oestrogens is complicated because so many different kinds of oestrogens are present, including oestrone sulfate (15%), equilin sulfate (25%), dihydroequilin sulfate (15%) and several other oestrogen sulfates. All of these oestrogens undergo metabolic conversions in the gastrointestinal tract and liver. Equilin sulfate and oestrone sulfate are the major components (approximately 40%) of the equine oestrogen preparation Premarin®, which is the most widely prescribed oestrogen used in therapy for post-menopausal women in the United States.

### 4.1.1 *Humans*

The absorption of oestradiol in humans has been studied extensively; however, the results are difficult to compare as different preparations of oestradiol and different routes of administration have been used.

Daily oral administration of oestradiol tablets results in large pulses of oestradiol and oestrone and exposes women to high concentrations of these compounds. Oral administration of the first oestradiol tablet, 2 mg micronized oestradiol, to 32 healthy post-menopausal women resulted in a maximal plasma oestradiol concentration of 1084 pg/mL 49 min after administration, which decreased rapidly during the subsequent 3 h. Progressive accumulation of oestradiol occurred until a steady state was reached. After the fifth tablet, the average concentration of oestradiol was about 418 pg/mL, which was 12 times greater than that found when a transdermal patch was used. The oestrone concentration reached a peak of 334 pg/mL 4.3 h after the first administration and reached a steady state

**Figure 4. Pathways for the metabolism and redox cycling of oestradiol, oestriol and oestrone**




Modified from Yager & Liehn (1996)

after the 14th daily administration. This average concentration of oestrone was 9.4 times greater than that found when a transdermal patch was used (Setnikar *et al*., 1996).

After a single oral dose of 2.0 mg/day oestradiol valerate to post-menopausal women, the maximal plasma concentrations were 0.96 nmol/L oestrone, 0.19 nmol/L oestradiol, 44.4 nmol/L oestrone sulfate, 0.6 nmol/L oestradiol sulfate and 0.19 nmol/L oestriol sulfate. The times to reach the maximal concentration were 5.2 h for oestrone, 3.2 h for oestradiol, 4.1 h for oestrone sulfate, 5.0 h for oestradiol sulfate and 8.8 h for oestriol sulfate (Aedo *et al*., 1990).

A comparison of the pharmacokinetic parameters of oral and sublingual administration of micronized oestradiol to post-menopausal women revealed that the time to the maximal concentration of oestradiol was significantly different by the two routes of administration, being 1 h or less for sublingual administration and 6.5–7.6 h for oral administration. The maximal plasma concentration, terminal half-life, area under the curve for the integral of the serum concentration over time (area under the curve) and oral clearance were also different with the two routes of administration. For example, after sublingual administration of 1 mg micronized oestradiol, the maximal plasma oestradiol concentration was 451 pg/mL, the terminal half-life was 18 h, the area under the curve was 2109 pg/mL per h and the oral clearance was 7.6 L/h per kg bw; after oral administration, these values were 34 pg/mL, 20.1 h, 823 pg/mL per h and 27.2 L/h per kg bw, respectively. The concentrations of oestrone were not dependent on route of administration. Sublingual administration resulted in a significantly lower ratio of oestrone to oestradiol than oral administration during the 24-h period (Price *et al*., 1997).

Because oestrogen penetrates normal skin easily (Jewelewicz, 1997), various preparations based on this property have been evaluated, including subcutaneous implants, vaginal creams and rings, percutaneous gels and transdermal therapeutic systems. Transdermal oestradiol provides physiological levels of oestradiol at a constant rate; the transdermal route avoids loss of drug by the hepatic first-pass effect and minimally affects hepatic protein metabolism. As a result, the oestradiol:oestrone ratios more closely resemble those of pre-menopausal women. Maximal serum concentrations of oestradiol are reached within 2–8 h of application of a transdermal system. When 50 µg/day transdermal therapy is used on a long-term basis, the mean steady-state serum concentrations can be 20–50 pg/mL. In a study of 100 µg/day transdermal therapy, the mean oestradiol concentration was 46–152 pg/mL. Within 24 h of removal of the transdermal delivery system, the plasma concentrations of oestradiol and oestrone and the urinary excretion of oestradiol and oestrone conjugates generally returned to pre-treatment levels (Balfour & Heel, 1990).

The pharmacokinetic profiles of oestradiol and oestrone have been reported in 16 healthy post-menopausal women after twice weekly applications for three weeks of an oestradiol transdermal patch, which contains 4 mg oestradiol and delivers 50 µg oestradiol daily. During the first application, oestradiol reached effective concentrations of 30 pg/mL or more 12 h after application; during the following five applications, the concentration remained constant at an average of 35 pg/mL. After removal of the patch, the concentration

returned to basal level within 12 h. The oestrone concentration reached a maximum of 48 pg/mL 41 h after the first application and then remained constant (Setnikar *et al*., 1996).

Seven days' use of patches containing 0.1 or 0.05 mg/day oestradiol resulted in peak average blood concentrations of 100 and 50 pg/mL, respectively. Values approximating 90% of the maximal level were achieved within 12 h after patch application and were maintained for up to 48 h. The mean steady-state blood concentrations over seven days were approximately 70 and 35 pg/mL, respectively. After removal of the patch, the concentrations fell to near baseline within 12 h (Gordon, 1995).

Subcutaneous administration of oestradiol pellets containing 25–200 μg pure crystalline oestradiol resulted in good bioavailability, as seen from the oestradiol:oestrone ratios, because minimal metabolism occurs in subcutaneous tissues (Jewelewicz, 1997). Pellets of 25 mg and 50 mg oestradiol produced serum concentrations of oestradiol of approximately 50–70 pg/mL and 100–120 pg/mL, respectively, for up to several months (Stumpf, 1990). Although the serum oestradiol concentrations were reported to be stable with this route of administration, 12 women of similar age and body weight in one small open study showed striking variations in hormone concentrations over 12 months of follow-up (Jewelewicz, 1997).

In a study of 24 women with vaginal atrophy treated daily with vaginal tablets containing 10 or 25 μg oestradiol, the oestradiol plasma concentrations reached 45 and 60 pmol/L after two weeks, respectively. The plasma oestrone concentration was unchanged by treatment (Johnston, 1996).

Oestradiol-containing polydimethylsiloxane rings inserted into the vagina release oestradiol continuously, and the substance is readily absorbed by the vaginal mucosa. Relatively constant serum concentrations of approximately 150 pg/mL oestradiol and oestrone were achieved for 21 days from a ring containing 400 mg oestradiol (Kuhl, 1990). The bioavailability of oestradiol from vaginal rings has been reported to be 13 ± 7% (range, 7–27%) in post-menopausal women (Gabrielsson *et al*., 1995).

After intramuscular injection of oestradiol esters in oily solution, the compound is released slowly from the primary depot at the injection site and/or from secondary depots in fat tissue (Kuhl, 1990).

Oral intake of 8 mg oestriol resulted in a maximum plasma concentration of 75 pg/mL unconjugated oestriol after 2 h, and the concentrations increased to up to 130 pg/mL after continued daily ingestion of 8 mg oestriol for 30 days, although the serum concentration of conjugated oestriol remained unaltered (Schiff *et al*., 1978). Orally administered oestriol was almost completely conjugated in the intestine to glucuronides (80–90%) and sulfates (10–20%); only 1–2% of the parent steroid reached the circulation (Kuhl, 1990).

Oestriol undergoes much less metabolism after vaginal application than after oral ingestion, and 20% of the dose appears as unconjugated steroid in the blood. At a dose of 0.5 mg, peak levels of 100–150 pg/mL were observed within 2 h. The maximal concentrations of oestriol after vaginal application of 0.5 mg were similar to those obtained after oral intake of 8–12 mg oestriol (Kuhl, 1990). There was no significant difference in the

plasma oestriol concentrations after vaginal administration of 1 mg and oral administration of 10 mg oestriol (Heimer, 1987). Daily administration of 1 g of a cream containing 500 µg oestriol to 11 post-menopausal women for eight weeks caused a mean rise in plasma oestriol from unmeasurable (< 35 pmol/L) to 87 pmol/L (Haspels *et al*., 1981). Treatment with a low dose of oestriol by the vaginal route may therefore induce systemic effects comparable to those achieved with high oral doses.

After a single oral dose of 2.5 mg/day piperazine oestrone sulfate to post-menopausal women, the maximal plasma concentrations were 1.3 nmol/L oestrone, 0.25 nmol/L oestradiol, 54 nmol/L oestrone sulfate, 0.9 nmol/L oestradiol sulfate and 0.23 nmol/L oestriol sulfate. The time to reach the maximal plasma concentration was 6.4 h for oestrone, 9.8 h for oestradiol, 4.4 h for oestrone sulfate, 6.5 h for oestradiol sulfate and 4.9 h for oestriol sulfate (Aedo *et al*., 1990).

Most of the available data on distribution are based on studies of intravenous administration. After intravenous administration of oestradiol to post-menopausal women, a high clearance (1.8 ± 0.6 L/min) and a low distribution volume (51 ± 28 L) were found. Oestrogens circulate in the blood bound to albumin (about 60%), sex hormone-binding globulin (about 38%), $\alpha_1$-glycoproteins and transcortin. Oestradiol binds weakly to albumin (low affinity/high capacity; plasma concentration, 40 g/L), about one-third is tightly bound to sex hormone-binding globulin (high affinity/low capacity) and a small fraction (< 3%) is 'free'. After intravenous administration, the distribution volume of oestradiol at steady state was only about 70 L, representing 1.5–2 times the total body water in fertile women. Its low level of distribution is consistent with its high level of binding to plasma proteins. The production of sex hormone-binding globulin is stimulated by increasing oestrogen concentration. Because of the high concentration of albumin (about five orders of magnitude higher than sex hormone-binding globulin) and its rapid dissociation, albumin may serve a more important regulating role (Gabrielsson *et al*., 1996).

After daily transdermal treatment of post-menopausal women with 0.1 mg oestradiol for three consecutive cycles, there was no significant difference in the distribution of oestradiol and oestrone between free and protein-bound oestrogen fractions in peripheral plasma (Jasonni *et al*., 1988).

Oestrone is not tightly bound to plasma proteins and therefore has a higher clearance rate than oestradiol. Oestrone sulfate is quantitatively the most important plasma oestrogen metabolite and is bound with high affinity to albumin, 90% circulating in bound form. Oestrone sulfate is considered to have a large, slowly metabolized reservoir, with 90% of its mass bound to albumin; accordingly, it has a low metabolic clearance rate (about 150 L/24 h) and low renal clearance (Anderson, 1993).

Few studies have addressed the accumulation and storage of oestradiol, oestrone and oestriol after exogenous administration. All three are distributed to various target and non-target organs through the systemic circulation but are also produced locally and accumulate in target tissues particularly rich in fat. Knowledge about the metabolism of oestradiol, oestrone and oestriol in humans has not advanced much since the last evaluation (IARC, 1979), except for hydroxylation of oestradiol.

The metabolic disposition of oestrogens includes oxidative metabolism (largely hydroxylation) and conjugative metabolism by glucuronidation, sulfonation and/or *O*-methylation (reviewed by Zhu & Conney, 1998). Oestradiol is converted to oestrone by a 17β-hydroxysteroid dehydrogenase; the oestrone produced is further metabolized to 16α-hydroxyoestrone and then to oestriol (Johnston, 1996). Hydroxylation of oestradiol at the 2 position is a major metabolic pathway in the liver (Kerlan *et al*., 1992). There are large inter-individual differences in oestradiol 2-hydroxylation in human liver samples, which may be reflected by differences in oestrogenic action. 4-Hydroxylation of oestradiol to a catechol is a minor pathway (usually < 15% of 2-hydroxylation) in the liver. Recent studies have shown that 4-hydroxylation of oestradiol is the dominant pathway of catechol oestrogen formation in human breast and uterus (Figure 4; Liehr *et al*., 1995; Liehr & Ricco, 1996). In humans, 4-hydroxylation of oestradiol is catalysed by the cytochrome P450 enzyme CYP1B1 (Hayes *et al*., 1996). Oestradiol and oestrone hydroxylated can undergo metabolic redox cycling *in vitro* to generate free radicals such as superoxide and the chemically reactive oestrogen semiquinone/quinone intermediates (Liehr & Roy, 1990). In the presence of fatty acid acyl-coenzyme A, oestradiol can be converted at the C-17 position to very lipophilic oestrogen fatty acid esters by enzymes present in liver and in oestrogen target organs such as breast and placenta (Adams *et al*., 1986).

Oestrone sulfate is the oestrogen found at the highest concentration in plasma and seems to constitute a storage form for circulating oestrogens. Oestrone sulfate can be hydrolysed to oestrone by arylsulfatases, which are widely distributed in human tissues (Rozenbaum, 1996).

There is a reversible equilibrium between oestradiol, oestrone and oestrone sulfate, which are interconverted by oestradiol dehydrogenase, sulfotransferase and aryl sulfatase. The two pathways of phase I oestrone inactivation are ring A metabolism, which produces catechol–oestrogens and is favoured in underweight women and hyperthyroid patients, and ring D metabolism, which leads to oestriol production and is increased in obese women and hypothyroid patients. Phase II metabolism involves the formation of several oestrogen conjugates; the sulfates circulate in high concentrations in the blood, and the glucuronides are excreted with the bile and in urine. After oral administration of micronized oestradiol, the serum oestrone:oestradiol ratio, which is 1:2 in fertile women and 2:1 in post-menopausal women, increased to 4:1. Prolonged percutaneous or transdermal treatment with oestradiol in conjunction with subcutaneous implantation of oestradiol pellets has been reported to lead to continuously elevated oestrogen levels and an oestrone:oestradiol ratio of 1:1 to 1:2 (Kuhl, 1990).

Conjugated equine oestrogens are hydrolysed to their active form in the gastrointestinal tract and also undergo considerable hepatic metabolism before entering the bloodstream in an active form (Ansbacher, 1993). Most sulfate esters are hydrolysed to free or unconjugated oestrogen by enzymes in the lower gut; the free oestrogen is absorbed by intestinal tissue, where it can be reconjugated with sulfate. Therefore, the oestrogen sulfate found in the bloodstream is not the same sulfate that was administered. The rate of dissolution is important because it influences where the active ingredients of the product

are released in the gastrointestinal tract, a factor which may affect the amounts of the oestrogen that are activated and the patterns of active and inactive metabolites. Equilin and equilenin are interconverted to 17β-dihydroequilin and 17β-dihydroequilenin and correspond to the interrelation between oestrone and oestradiol. As in the case of natural oestrogen in women, there is an equilibrium between equilin, equilenin and their metabolites and the respective sulfates (Kuhl, 1990).

The sulfate esters of equilin, oestrone and oestradiol do not bind sex hormone-binding globulin; however, equilin sulfate and oestrone sulfate interact with serum albumin with high affinity ($0.9–1.1 \times 10^5$/mol per L). Up to 74% of total equilin sulfate and 85–90% of oestrone sulfate were bound to serum albumin (Bhavnani, 1998). The peak concentration of equilin sulfate is found after 4 h. Equilin is rapidly absorbed and converted to 17β-dihydroequilin. The volume of distribution at steady state was 6 ± 0.5 L for 17β-dihydroequilin sulfate, 23 ± 1.3 L for 17β-dihydroequilin and 12.4 ± 1.6 L for equilin sulfate (O'Connell, 1995).

Six healthy post-menopausal women were seen each month during a six-month trial of cyclic therapy with conjugated equine oestrogens (Premarin®), ingested at 1.25 mg per day for 21 days followed by seven days without therapy. The average serum concentrations in samples taken within 2 h of the last ingestion of drug in a given cycle were 1850 pg/mL unconjugated equilin, 162 pg/mL oestrone and 106 pg/mL oestradiol. Three months after completion of therapy, the oestrone and oestradiol concentrations had returned to pre-treatment levels, but equilin was still detected in serum at a concentration of 144 pg/mL in all three of the women who were investigated (Whittaker *et al.*, 1980).

After administration of $^3$H-equilin sulfate and $^3$H-17β-dihydroequilin sulfate to post-menopausal women, less than 50% of the administered dose was excreted in the urine. The majority (63–74%) of the radiolabelled metabolites excreted were in the form of glucuronides, whereas 16–17% were found as sulfates and 1–2% in the unconjugated fractions (Bhavnani, 1998). About 40–50% of the radiolabel from injected oestradiol is excreted in the bile (Sandberg & Slaunwhite, 1957).

These studies show that the bioavailability of oestrone, oestradiol and oestriol depends on the formulation, route of exposure and dose of oestradiol administered. Generalizations cannot be made about the disposition of oestrogen because of differences in the regimens, products and route of exposure and other factors such as age and inter- and intra-individual variations. It can be concluded, however, that transdermal administration by patch, percutaneous administration or transvaginal administration allow more circulating oestrogen for a longer time than oral administration. Comparative trials of various oestrogen products are urgently needed, keeping in consideration the route of exposure and the composition of the different products, to allow quantitative evaluation of the disposition of oestrogens.

### 4.1.2 *Experimental systems*

Few studies have been carried out in experimental systems on the disposition of oestrone, oestradiol and oestriol products since the previous evaluation (IARC, 1979), and

limited experimental studies are available on the absorption, distribution and excretion of oestradiol. Oestrone, oestradiol and oestriol undergo various phase I and phase II metabolic reactions in humans and animals (reviewed by Zhu & Conney, 1998). During phase I metabolism, oestrone, oestradiol and oestriol serve as substrates for aromatic hydroxylation, and these reactions are catalysed by cytochrome P450 enzymes. Oestrone and oestradiol are converted to 2- and 4-hydroxyoestrone and 2- and 4-hydroxyoestradiol, respectively, the ratio of hydroxylated products depending on the target tissues and animal species. Oestradiol and its metabolites serve as substrates for sulfation, methylation and glutathione conjugation. The metabolism depends on the species, strain and sex of the experimental animals and on the experimental conditions.

In the early 1970s and 1980s, it was postulated that oestradiol is converted to reactive intermediates during its metabolism, and direct evidence of reactive metabolites of oestradiol has now been obtained (Liehr *et al*., 1986b; Roy *et al*., 1991). Catechol oestrogens undergo microsomal cytochrome P450-mediated redox cycling reactions (Liehr *et al*., 1986b; Liehr & Roy, 1990; Roy *et al*., 1991), resulting in the formation of reactive metabolites. Both catechols of oestradiol are converted to their respective quinones in the presence of metabolic activation systems. Nuclei can also catalyse redox cycling of oestrogens (Roy & Thomas, 1994). *In vitro*, oestradiol in the presence of a metabolic activation system can be converted to DNA-binding oestrogen quinone metabolite(s) (Liehr *et al*., 1993), but DNA binding has not been detected *in vivo*.

Oestrone, oestradiol and oestriol are excreted in the bile as glucuronides and undergo enterohepatic recirculation. Their glucuronides are hydrolysed in the intestine, and unconjugated oestradiol or oestrone is reabsorbed from the intestine by enterohepatic cycling (Zhu & Conney, 1998).

Topical application of radiolabelled oestradiol to shaved skin of the dorsal neck of rats at a dose of 30.1, 120.4 or 301 pmol/cm$^2$ and autoradiography revealed the presence of oestradiol in epidermis, sebaceous glands, dermal papillae of hair and fibroblasts 2 h after application. A high concentration and retention of oestradiol in sebaceous glands was observed for more than 24 h, suggesting that sebaceous glands serve as a second storage site for oestradiol (Bidmon *et al*., 1990). An effect of dose and the area of topical application of oestradiol has also been observed in a hairless strain of rats. After a single dose of 50 nmol applied topically, the bioavailability, determined by urinary and faecal excretion of radiolabel after four days, was not affected by the area of the application surface. When the applied doses were increased from 50 to 1000 and 10 000 nmol, the percentage of percutaneous absorption decreased with reduction in the area of application (Chanez *et al*., 1989). Nasal administration of oestradiol to rats resulted in significantly higher blood levels than after intraduodenal administration, the bioavailability being 50% after a dose of 5 μg, 71% after 10 μg and 84% after 20 μg/rat compared with 2–5% via the intraduodenal route for the same doses (Bawarshi-Nassar *et al*., 1989). In rats and rabbits, nasal administration of oestradiol with dimethyl-β-cyclodextrin as a solubilizer and absorption enhancer resulted in significantly more absorption of the oestrogen than when it was given in suspension. Nasal administration of oestradiol–dimethyl-β-cyclodextrin

resulted in an absolute bioavailability of 94.6% in rabbits and 67.2% in rats in relation to an intravenous injection (Hermens *et al*., 1990).

In a study in rats given oestradiol–bisphosphonate conjugates with different esterase-sensitive linkers between the two molecular moieties, the conjugate with the low-cleavage resistance doubled the serum half-life of oestradiol (3.78 h), and the high-cleavage resistance conjugate resulted in a serum half-life approximately four times higher (8.36 h) than that of free oestradiol (Bauss *et al*., 1996).

After administration of oestriol to rats, glucuronides and sulfates of 16-keto-oestradiol and of 2- and 3-methyl esters of 2-hydroxyoestriol and 2-hydroxy-16-ketooestradiol were excreted in the bile (Bolt, 1979).

Oestradiol represented only 6% of the total oestrogen detected in the hepatic portal vein after oestradiol was placed in the stomach of a prepubertal pig; thus, most of the oestradiol was converted or conjugated before entering the hepatic portal vein. The blood concentrations of oestradiol glucuronide, oestrone glucuronide and oestrone sulfate but not of oestradiol or oestrone in the jugular vein rose and remained elevated for several hours, indicating that oestradiol and oestrone are completely converted and/or removed by the liver (Ruoff & Dziuk, 1994a).

In a comparison of the serum and tissue concentrations of oestradiol in fertile female and in castrated male Syrian golden hamsters, oestradiol pellets (20 mg) were implanted into the shoulder region of groups of four to six hamsters every 45 days to maintain the hormone concentration, and the animals were killed after 15 days and at 30-day intervals. The average serum oestradiol concentration in the cycling female hamsters was 79 pg/mL on days 1–2 and 311 pg/mL on days 3–4, attaining a maximum of 358 pg/mL on day 4 of the cycle. The concentrations on days 3–4 of the cycle were threefold higher than those on day 1 in uterine tissue, twofold higher in renal tissue and 2.6-fold higher in hepatic tissue. As was to be expected, the serum oestradiol concentrations of untreated castrated male hamsters did not vary appreciably over the six months of the study, and the average was about 32 pg/mL. Under conditions that produced essentially 100% renal tumour incidence, the serum oestradiol concentration rose rapidly to an average of 71-fold the untreated level. A steady-state serum concentration of 2400–2700 pg/mL was maintained during 45–180 days of continuous oestrogen treatment. The renal concentration of oestradiol in hamsters given this hormone rose by an average of only 5.4-fold between days 15 and 180 of treatment, and the serum concentrations were 5.7- to 8.0-fold higher than those in cycling female hamsters on days 3 and 4, with, however, no apparent effect on weight or mortality rate (Li *et al*. 1994).

A 10-mg dose of crystalline oestradiol placed in the rectum of prepubertal gilts resulted in increased concentrations of oestradiol, oestrone, oestradiol glucuronide, oestrone glucuronide and oestrone sulfate in the hepatic portal vein within 30 min, and the concentrations remained elevated for several hours (Ruoff & Dziuk, 1994b). After oestradiol was placed in the stomach of the prepubertal gilts, the concentrations of oestradiol, oestrone, oestradiol glucuronide, oestrone glucuronide and oestrone sulfate in the hepatic portal vein rose within 5 min and remained elevated for several hours (Ruoff &

Dziuk, 1994a). Most of the conjugated metabolites in liver and kidney of cattle are glucuronides (85–95%) (Kaltenbach *et al*., 1976).

In pregnant rhesus monkeys, oestradiol was eliminated from the maternal circulation principally by conversion to glucuronide conjugates (Hill *et al.*, 1980; Slikker *et al*., 1982). After pulse injection of $^{3}$H-oestrone sulfate to adult female rhesus monkeys, the initial volume of distribution was 4.6 ± 0.9 L, and the metabolic clearance rate was 42 ± 2.9 L/day. Infusion of $^{3}$H-oestrone sulfate or $^{14}$C-oestrone resulted in a metabolic clearance rate of 67.5 ± 8.3 L/day. The conversion ratio of oestradiol to oestrone sulfate was 0.054 ± 0.016; the interconversion value was 43.6 ± 3.4% for oestrone sulfate to oestrone and 33.5 ± 6.6% for oestrone to oestrone sulfate. Thus, oestrone sulfate is cleared slowly and is converted to both oestrone and oestradiol (Longcope *et al*., 1994).

The disposition of equilin sulfate was determined in female dogs receiving 2.5 mg/kg bw $^{3}$H-equilin sulfate orally. The drug was rapidly absorbed (time to reach maximal concentration in plasma, 1 h) and had a moderate half-life (16.3 ± 9.6 h) in plasma. An average of 26.7 ± 4.4% of the administered radiolabel was excreted in urine. When $^{3}$H-equilin sulfate was administered as part of a conjugated equine oestrogen preparation, a lower peak concentration, a lower area under the curve, a longer terminal half-life and a lower elimination percentage in urine were observed, indicating that the absorption of equilin sulfate was altered by other components in the preparation. Both plasma and urine contained equilin, equilenin, 17β-dihydroequilenin, 17β-dihydroequilin, 17α-dihydroequilenin and 17α-dihydroequilin. 17β-Dihydroequilin and equilin were the two major chromatographic peaks in plasma, whereas 17β-dihydroequilenin and 17β-dihydroequilin were the major metabolites in urine. The reduction of the 17-keto group and aromatization of ring-B are the major metabolic pathways of equilin in dogs (Chandrasekaran *et al*., 1995).

Uptake of oestrone sulfate has been reported by isolated rat hepatocytes. Accumulation in the cell remained linear with time up to 1 min and then began to decrease. The $K_m$ was 16 ± 6 μmol/L, and the $V_m$ was 0.85 ± 0.56 nmol/min per $10^6$ cells. The uptake of oestrone sulfate involves a $Na^+$- and energy-dependent transport protein and a $Na^+$-independent anionic transport or multiple organic anion transport (Hassen *et al.*, 1996).

4-Hydroxylation of equilenin was reported in hamster liver microsomes (Sarabia *et al*., 1997). *In vitro*, 4-hydroxyequilenin can participate in redox cycling, and its quinone metabolite (4-hydroxyequilenin-*ortho*-quinone) is more reactive than 4-hydroxyoestrone-*ortho*-quinone (Shen *et al.*, 1997).

## 4.2 Receptor-mediated effects

### 4.2.1 *Humans*

In hyperplastic endometrial samples from four women who had been exposed to oestrogen [not further specified], the expression of keratinocyte growth factor mRNA was suppressed to the concentrations found in endometrial samples from unexposed women during the late proliferative phase of the menstrual cycle, whereas the expression of keratinocyte growth factor receptor mRNA was increased by approximately 35% over the enhanced levels found in the same control women (Siegfried *et al*., 1995).

In endometrial biopsy samples from post-menopausal women who had received 1.25 mg Premarin® daily for at least three months, the in-vitro DNA labelling index (incorporation of tritiated thymidine) in glandular epithelial cells and the nuclear oestradiol receptor content were increased and were similar to those found in proliferative-phase endometrium from eight unexposed women. The number of tritiated thymidine-labelled cells per microscopic high-power field was increased threefold by the treatment (four women) in comparison with the pre-menopausal control values (eight women) (Siddle *et al*., 1982).

Groups of post-menopausal women continuously received either oral doses of 2 mg per day oestradiol valerate, 1.5 mg per day oestropipate, 0.625 or 1.25 mg per day conjugated equine oestrogens (Premarin®), 50-mg oestradiol implants or 5 g of a skin cream containing 3 mg oestradiol; most women received a progestogen during the last 7–10 days of each month. Endometrial biopsy samples were obtained, and the receptor content was measured. The content of soluble progesterone receptor was not affected in 13 women taking Premarin® when compared with the level in proliferative-phase endometrium from 12 unexposed women. The nuclear oestradiol receptor content was slightly elevated (by 30%) in the endometrial samples from the 15 women who had received the high dose of Premarin® (1.25 mg/day) as compared with the level in proliferative-phase endometrium from 16 unexposed women. The percentage of endometrial glandular cells that had incorporated tritiated thymidine *in vitro*, examined only in cells from five women given 1.25 mg per day Premarin®, appeared to be minimally elevated (by approximately 10%) over the labelling index observed in proliferative-phase endometrium of 12 women; the endometrial oestrogen receptor content was increased by approximately 25% in these samples. All of the studies indicated a slight increase in cell proliferation in Premarin®-exposed women (Whitehead *et al*., 1981).

The effect of oral treatment for 90 days with 0.2 mg/day of a constituent of conjugated equine oestrogens, 17α-dihydroequilin sulfate, or 1.25 mg per day oestrone sulfate or a combination of these two steroids was examined in groups of seven women in whom menopause had been surgically induced. The serum concentration of sex hormone-binding globulin, measured as an indicator of oestrogenic activity, was increased after the oestrone sulfate treatment, by 20% after 30 days and by 60% after 90 days. Exposure to 17α-dihydroequilin, however, caused a 21 and 12% reduction of this parameter after these time intervals, whereas the combined treatment synergistically increased the concentrations of sex hormone-binding globulin by approximately 100% after 30 or 90 days (Wilcox *et al*., 1996). Ingestion by post-menopausal women of conjugated equine oestrogens at doses of 0.9–1.25 mg/day significantly increased the cortisol-binding capacity of transcortin, whereas lower doses of conjugated equine oestrogens (0.3 and 0.6 mg/day) did not (Schwartz *et al*., 1983).

Post-menopausal women received oral doses of conjugated equine oestrogens (0.625 mg per day Premarin®) for 24 days each month or transdermal doses of oestradiol via adhesive patches delivering 0.05 mg/day every fourth day for the first 24 days of each month. Some women also received a progestogen (dydrogesterone) during the last 12 days

of each month. The serum concentrations of insulin-like growth factor-I were decreased as compared with the pre-treatment levels in the Premarin®-treated group only. The growth hormone and sex hormone-binding globulin concentrations were increased in this group but not in the women receiving transdermal oestrogen (Campagnoli *et al*., 1993).

4.2.2 *Experimental systems*

(*a*) *Conjugated oestrogens*

The equine oestrogens equilin and equilenin bound to the oestrogen receptor with low affinity (less than oestrone) when examined by displacement of radiolabelled 17β-dihydroequilin in rat and human uterine tissue (Bhavnani & Woolever, 1991).

Oestrone sulfate at concentrations of $10^{-7}$ mol/L and higher stimulated the growth of MCF-7 human breast cancer cells determined after six days; however, when the mitotic index was used as the indicator of cell proliferation, stimulation was already maximal at a concentration of $10^{-10}$ mol/L, reaching the same level as that achieved with oestradiol at $10^{-12}$–$10^{-11}$ mol/L. This effect of oestrone sulfate was inhibited by simultaneous exposure of the cells to the pure anti-oestrogen ICI164,384. Induction of progesterone receptor or production of the pS2 protein, both indicators of cellular oestrogenic effects, occurred only at a concentration of $10^{-6}$ mol/L and was weaker than that after oestradiol treatment. Three days of treatment of MCF-7 cells with $10^{-7}$ or $10^{-9}$ mol/L oestrone sulfate resulted in concentrations in the medium of $4.4 \times 10^{-9}$ mol/L oestrone and $1.0 \times 10^{-9}$ mol/L oestradiol (average of two experiments at the high dose) in comparison with $3.3 \times 10^{-11}$ mol/L oestrone and $4.4 \times 10^{-12}$ mol/L oestradiol (at the low dose). Importantly, treatment of MCF-7 cells with $10^{-7}$ mol/L oestrone sulfate resulted in considerable accumulation in isolated nuclei of free oestrone (560 pg/mg DNA) and oestradiol (180 pg/mg DNA) but very litte oestrone sulfate (13 pg/mg DNA) (Santner *et al*., 1993).

Twenty-two adult female cynomolgus monkeys that had undergone surgical menopause were given Premarin® in a diet in which 40% of the calories were from fat; 26 animals received control diet. The daily dose of Premarin® was approximately 7.2 μg per animal for the first eight months of the experiment and 166 μg per animal for the subsequent duration of the 30-month study; the latter dose was stated by the authors to be equivalent to a human dose of 0.625 mg per day. The oestrogen treatment increased the concentration of circulating oestradiol from 5 to 167 pg/mL, increased the thickness of the mammary tissue by 50% and significantly enlarged the estimated surface area of lobular tissue. The mean percentage of epithelial breast cells that stained for Ki-67 MIB-1 antibody (a marker of cell proliferation) was increased from 2.5 to 5.4% in alveoli, from 0.6 to 2.1% in terminal ducts and from 1.2 to 3.0% in major mammary ducts. The mean percentage of epithelial breast cells that stained for progesterone receptors (with antibody techniques) was increased four- to sixfold in these mammary glands, but the percentage of cells that stained for oestrogen receptor was not significantly affected. Oestrogen treatment induced mammary gland hyperplasia in 9/22 of the monkeys as compared with none of the control group (Cline *et al*., 1996).

Hydrolysed Premarin® stimulated cell proliferation in primary cultures of renal proximal tubular cells isolated from castrated male Syrian golden hamsters at concentrations of $10^{-9}$ and $10^{-8}$ mol/L, with no effect at either higher or lower concentrations. Treatment of these hamsters with pellets that released 111 ± 11 μg hydrolysed Premarin® per animal per day resulted in a 100% tumour incidence in the kidney within approximately nine months (Li *et al*., 1995).

Equilin bound to sex hormone-binding globulin, displacing 5α-dihydrotestosterone with an affinity that was 50% that of oestradiol and 5.6% that of testosterone (Pan *et al*., 1985).

(*b*) *Oestradiol*

Oestradiol is the natural ligand for the oestrogen receptor. It drives the oestrogen responses of the uterus and mammary gland, which are typical oestrogen-sensitive organs, and in many other tissues. Classical responses to oestradiol mediated by the oestrogen receptor include uterine growth in immature or ovariectomized rodents and transcriptional activation of the progesterone receptor (see, e.g., Musgrove & Sutherland, 1997; Rutanen, 1997). The response of these tissues to oestradiol and the effects observed in cells and with oestrogen-responsive reporter gene constructs, are usually taken as a standard against which the oestrogenicity of other compounds is measured (see, e.g., Jeng *et al*., 1992; Katzenellenbogen *et al*., 1993; Parker, 1995; Kuiper *et al*., 1996). Similarly, displacement of radiolabelled oestradiol is the standard by which binding affinity to the oestrogen receptor is determined. The nature of these oestrogen responses depends mainly on the stage of development of the tissues and the age of the organism. Two high-affinity–low-capacity forms of the receptor have been identified: the oestrogen receptors α and β (Koike *et al*., 1987; Parker, 1995; Kuiper *et al*., 1996). Both types are widely distributed and have been isolated from several mammalian tissues (Greene *et al*., 1986; Krust *et al*., 1986). The role of the oestrogen receptor-β is still largely unclear, and almost all of the reports in the literature about oestrogen binding, oestrogen receptor–ligand interactions and transcriptional regulation by the oestrogen receptor–ligand complex pertain to the oestrogen receptor-α. The oestrogen responses that have been observed in many tissues and cells are probably, however, a result of the compound involvement of the oestrogen receptors α and β, although the relative contribution of each is still unknown. Anti-oestrogens, such as tamoxifen, which also has weak agonist activity, and pure anti-oestrogens, such as ICI164,384 and ICI182,780, compete with oestradiol for oestrogen receptor binding and are typically used to demonstrate that oestrogenic responses are mediated by this receptor (Jeng *et al*., 1992; McDonnell *et al*., 1995). In the context of this monograph, the responses to exogenous oestradiol in various tissues of pre- and post-menopausal women and the effects of oestradiol in appropriate animal or in-vitro models are the most relevant and are summarized here.

Oestradiol strongly induced growth of both MCF-7 and T47D human breast cancer cell lines and other human breast cancer cell lines that contain oestrogen receptors, but it did not stimulate growth of oestrogen receptor-negative breast cancer cell lines (Jeng

*et al*., 1992; Catherino & Jordan, 1995; Schoonen *et al*., 1995a,b). In MCF-7 cells, the growth-stimulating effect of oestradiol was already observed at a concentration of $10^{-12}$ mol/L; it increased to a maximum at a concentration of about $10^{-10}$ mol/L and decreased at concentrations higher than $10^{-9}$ mol/L (Jeng *et al*., 1992). These growth-stimulating effects were abolished not only by anti-oestrogens such as tamoxifen and ICI182,780 but also by anti-progestogens such as RU486 (Catherino & Jordan, 1995; Schoonen *et al*., 1995a,b). The effects were demonstrated in experiments performed with breast cancer cell lines grown in phenol red-free medium which contained steroid-free (dextran-coated charcoal-stripped) serum (Jeng *et al*., 1992; Schoonen *et al*., 1995a,b).

At concentrations that stimulate growth of human breast cancer cells, oestradiol induced expression of oestrogen-responsive genes, such as the *pS2* gene, at the transcriptional level (Brown *et al*., 1984; Kalkhoven *et al*., 1994) and *trans*-activated oestradiol-responsive reporter constructs containing oestrogen response elements in oestrogen receptor-positive cells (Jeng *et al*., 1992; Kalkhoven *et al*., 1994; Catherino & Jordan, 1995). These effects were inhibited by anti-oestrogens. Most of the effects of oestradiol are believed to be mediated by triggering of the secretion of growth factors by autocrine and paracrine action (Lippman & Dickson, 1989). Transforming growth factor (TGF)-α and epidermal growth factor are the most extensively studied mediators of the cell growth-stimulating effects of oestradiol in breast and uterine tissues (see for reviews Boyd, 1996; McLachlan & Newbold, 1996; Snedeker & Diaugustine, 1996). Both up-regulation of these growth-enhancing factors and down-regulation of growth-inhibiting factors may be involved in the action of oestradiol. For example, oestradiol was shown to affect the production of the growth inhibitor TGF-β by human breast cancer cells (Knabbe *et al*., 1987; Arrick *et al*., 1990). It markedly down-regulated the mRNA expression of growth-inhibiting TGF-β2 and -β3 in MCF-7 cells at a concentration of oestradiol that stimulated the growth of these cells ($10^{-8}$ mol/L), but it did not affect the mRNA expression of TGF-β1 (Arrick *et al*., 1990; Jeng & Jordan, 1991). Other cytokines that are not under oestrogen regulation may also influence the growth of breast cancer cells, but oestradiol may affect the responsiveness of the cells to these factors. For example, a six-day exposure of oestrogen receptor-positive ZR-75-1 human breast cancer cells to $10^{-9}$ mol/L oestradiol reduced by 20–40% the expression of binding sites for interferon, which has anti-proliferative activity for these cells (Martin *et al*., 1991).

Oestradiol at $10^{-10}$–$10^{-9}$ mol/L increased the reductive activity of 17β-hydroxysteroid oxidoreductase [17-hydroxysteroid dehydrogenase] in an oestrogen- and progestogen-sensitive MCF-7 cell line in phenol red-free medium (Coldham & James, 1990). This activity may be involved in the mechanism of breast cancer cell growth stimulation through the induction of oestradiol formation.

Oestradiol at concentrations of $5 \times 10^{-12}$–$10^{-7}$ mol/L stimulated 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; Botella *et al*., 1995). This assay has been used as a marker for oestrogenic activity in endometrial cells.

Oestradiol at a concentration of $10^{-8}$ mol/L increased anchorage-independent growth of an SV40-immortalized human endometrial stromal cell line by almost 80% (Xu *et al*., 1995).

Oestradiol given subcutaneously to ovariectomized rats at a dose of 200 μg/kg bw per day for four days slightly decreased the gene expression of mitogen-activated protein kinase (MAPK) and increased MAPK tyrosine phosphorylation and membrane-associated MAPK enzymatic activity in uterine smooth-muscle tissue (Ruzycky, 1996). In the same model, oestradiol increased the membrane-associated protein expression of seven protein kinase C isozymes by 1.5–2.25-fold, whereas no such change occurred in cardiac muscle. These studies indicate that MAPK and protein kinase C play a role in two of the signal transduction pathways that regulate cell proliferation and are affected by oestradiol (Ruzycky & Kulick, 1996).

Groups of 10 female rats received 20 g DMBA by gavage over two weeks to induce mammary tumours. Ovariectomy suppressed MAPK expression in these tumours, and daily subcutaneous injections of oestradiol (10 μg/rat) induced activation of this enzyme in tumours in ovariectomized rats (Koibuchi *et al*., 1997).

In ovariectomized BALB/c mice, a single subcutaneous dose of 20 μg oestradiol 3-benzoate induced an approximately threefold induction of mRNA expression of the RXRα and RARγ retinoic acid receptor subtypes in cervical tissue within 0.5 and 4 h, respectively (Celli *et al*., 1996).

Treatment of cultures of normal human endometrial stromal cells with $10^{-8}$ mol/L oestradiol with or without $10^{-7}$ mol/L medroxyprogesterone acetate increased mRNA expression of vascular endothelial growth factor by 4.7- and 3.1-fold, respectively, over control values (Shifren *et al*., 1996); however, in ovariectomized nude mice carrying a human endometrial carcinoma xenograft, implantation of oestradiol pellets that maintained serum levels of this steroid at 200–300 pg/mL did not alter the expression of vascular endothelial growth factor in the tumour tissue (Kim *et al*., 1996). Oestradiol did not alter the secretion of vascular endothelial growth factor by T47D human breast cancer cells (Hyder *et al*., 1998) and had no effect on angiogenesis induction by basic fibroblast growth factor or TGF-α in rabbit cornea *in vitro* (Yamamoto *et al*., 1994).

Oestradiol decreased by approximately 50–60% the growth of decidual endothelial cells derived from human endometrium at concentrations of 0.5 and 2.5 ng/mL, increased the growth of these cells by approximately 30% at 5 ng/mL and had no effect at concentrations of 10 ng/mL and higher (Peek *et al*., 1995).

Oestradiol at concentrations above $10^{-10}$ mol/L stimulated the migration of human endometrial cancer cells (Ishikawa, HEC-1 or HHUA cells) through an artificial basement membrane and suppressed the mRNA expression of the cell adhesion-related molecules E-cadherin and α- and β-catenin in Ishikawa cells (Fujimoto *et al*., 1996a,b). At concentrations of $10^{-10}$ mol/L and higher, oestradiol up-regulated the mRNA expression of fibroblast growth factors-1 and -2 but not of fibroblast growth factor-4 in Ishikawa human endometrial cancer cells, with a maximal effect at $10^{-8}$ mol/L (Fujimoto *et al*., 1997). In contrast, no effect of oestradiol was found on endometrial adenocarcinoma SNG-M cells

in migration and invasion assays, which involve cell growth along a fibronectin gradient, or on their growth or locomotion as determined in a monolayer wounding model *in vitro*. The secretion of matrix metalloproteinases and stromelysin by these cells was not affected (Ueda *et al*., 1996).

Oestradiol has been shown to induce liver cell growth, increasing both liver weight and hepatic DNA content in female Wistar rats when given by subcutaneous injection (1–200 μg/kg bw per day) or in the diet (30–300 μg/kg bw per day) for seven days. The relationship between dose and response for these two parameters was approximately linear over the range of subcutaneous doses (Ochs *et al*., 1986; Schulte-Hermann *et al*., 1988). Oral administration of oestradiol was less effective than subcutaneous injection (Ochs *et al*., 1986).

Oestradiol at $3 \times 10^{-5}$ mol/L for 48 h induced a two- to threefold increase in tritiated thymidine incorporation into DNA of cultured primary female rat hepatocytes. Although oestradiol by itself thus appeared to have only weak mitogenic effects on primary rat hepatocytes, it strongly enhanced the induction of hepatic DNA synthesis by epidermal growth factor or TGF-α (Ni & Yager, 1994a,b). Epidermal growth factor-induced growth of male rat hepatocytes, however, was inhibited by concomitant oestradiol treatment at concentrations of $2.5 \times 10^{-6}$–$10^{-5}$ mol/L (Francavilla *et al*., 1989), suggesting marked sex differences in the mitogenic effects of epidermal growth factor and oestrogens on rat liver. Differences in culture medium composition may also have contributed to these discrepancies (Yager & Liehr, 1996).

Within 6–12 h after oestradiol was given at a dose of 5 mg/kg bw to castrated Syrian golden hamsters by intraperitoneal injection, renal ornithine decarboxylase activities were increased threefold above the control level. Similarly, in hamsters that received subcutaneous implants of pellets of 20 mg oestradiol to maintain chronically high oestrogen levels, the renal activity of ornithine decarboxylase was 1.5–1.9 times the corresponding activity in control animals after 60–80 days of treatment. With a series of oestrogen analogues, there was a direct correlation between the increase in renal ornithine decarboxylase activity *in vivo* and binding to renal oestradiol receptor sites *in vitro*. The concentrations of the polyamines putrescine, spermidine and spermine in hamster kidney all declined during the 180-day experimental period (Nawata *et al*., 1981).

### (*c*) *Oestriol*

Oestriol bound with low affinity to oestrogen receptors in calf, rat and human uterine tissue and in Ishikawa human endometrial cancer cells (Katzenellenbogen, 1984; Lubahn *et al*., 1985; Botella *et al*., 1995), with a relative binding affinity about 5–12% that of oestradiol (Batra *et al*., 1984; Katzenellenbogen, 1984; Botella *et al*., 1995).

Incorporation of tritiated thymidine by primary cultures of hepatocytes from female Fischer 344 or Lewis rats was minimally (1.5-fold) stimulated by addition of oestriol to the medium for 48 h at a concentration of $3 \times 10^{-5}$ mol/L. Simultaneous addition of 15 ng/mL TGF-α stimulated DNA synthesis in these cells by approximately 100-fold in

a synergistic fashion, while TGF-α alone at this concentration stimulated DNA synthesis by 54-fold (Ni & Yager, 1994a,b).

(*d*) *Oestrone*

Oestrone bound with low affinity to the oestrogen receptor in calf, rat and human uterine tissue (Lubahn *et al*., 1985; Bhavnani & Woolever, 1991) and in Ishikawa human endometrial cancer cells (Botella *et al*., 1995). Its relative binding affinity to the receptor in human endometrial cytosol is 2–10% that of oestradiol (Lubahn *et al*., 1985; Bhavnani & Woolever, 1991; Botella *et al*., 1995).

Oestrone stimulated the incorporation of tritiated thymidine into the DNA of MCF-7 human breast cancer cells at concentrations of $10^{-11}$ mol/L and higher, reaching a plateau of stimulation at $10^{-9}$ mol/L; this was approximately 20% lower than the maximal stimulation achieved by oestradiol at a concentration of $10^{-10}$ mol/L (Kitawaki *et al*., 1992). Oestrone treatment of MCF-7 human breast cancer cells stimulated their growth, as seen from the increase in cell number, over a six-day period at concentrations of $10^{-10}$ mol/L and higher, reaching a maximum at between $10^{-9}$ and $10^{-8}$ mol/L. When the mitotic index was used as an indicator of cell proliferation, however, stimulation was already maximal at an oestrone concentration of $10^{-13}$ mol/L and was similar to that achieved by oestradiol at $10^{-11}$ mol/L. This effect of oestrone was inhibited by simultaneous exposure of the cells to the pure anti-oestrogen ICI164,384. Three days of treatment of MCF-7 cells with $10^{-8}$ mol/L oestrone resulted in a concentration of $3.3 \times 10^{-9}$ mol/L oestradiol in the culture medium (Santner *et al*., 1993).

Oestrone given to female Sprague Dawley rats after ovariectomy at a subcutaneous dose of 1 μg/rat twice daily stimulated the growth of DMBA-induced mammary tumours by 225% after 65 days of treatment; this effect was abolished by simultaneous administration of an anti-oestrogen, EM-800, at 2.5 mg/kg bw per day or medroxyprogesterone acetate at 1 mg/rat subcutaneously twice daily (Luo *et al*., 1997).

Incorporation of tritiated thymidine by cultured primary hepatocytes from female Fischer 344 or Lewis rats was stimulated threefold by addition of oestrone to the medium for 48 h at a concentration of $3 \times 10^{-5}$ mol/L. Simultaneous addition of 15 ng/mL TGF-α stimulated DNA synthesis by approximately 100-fold in a synergistic fashion, while TGF-α alone at this concentration stimulated DNA synthesis by 54-fold (Ni & Yager, 1994b).

Oestrone at concentrations of $10^{-10}$–$10^{-8}$ mol/L stimulated cell proliferation in primary cultures of renal proximal tubular cells isolated from castrated male Syrian hamsters, being maximally effective at $10^{-9}$ mol/L and not effective at higher or lower concentrations. Treatment of these hamsters with pellets that released 111 ± 11 μg oestrone per animal per day resulted in a 100% tumour incidence in the kidney in approximately nine months (Li *et al*. (1995).

Oestrone bound to sex hormone-binding globulin, displacing 5α-dihydrotestosterone with an affinity that was 25% that of oestradiol and 1.8% that of testosterone (Pan *et al*., 1985).

## 4.3 Genetic and related effects

Receptor mechanisms are fundamental to the responses to oestrogens (King, 1991), although non-receptor mechanisms may also be important (Duval *et al.*, 1983; Yager & Liehr, 1996).

### 4.3.1 *Humans*

No data were available on the genetic effects of unopposed oestrogens in humans.

### 4.3.2 *Experimental systems*

The genetic and related effects of oestrogens are summarized in Table 14.

In one study, oestradiol decreased the formation of single- and double-strand breaks in ΦX-174 RFI DNA induced by hydrogen peroxide alone or with $Cu^{2+}$ (Tang & Subbiah, 1996). Gene mutations were not induced in *Salmonella typhimurium* by oestradiol. DNA strand breaks were not induced by oestradiol in rat hepatocytes or hamster ovary cells in the absence or presence of a metabolic activation system. Oestradiol weakly induced DNA breakage in mouse brain cells. It did not induce DNA repair in a mouse mammary cell line and did not give rise to unscheduled DNA synthesis in hamster embryo cells. It did not induce gene mutations at either the *hprt* or $Na^+/K^+$ ATPase loci, or sister chromatid exchange or chromosomal aberrations in hamster embryo cells, whereas the formation of micronuclei was increased in these cells in a single study. In several studies, aneuploidy was induced in hamster embryo cells, male hamster cells and human foreskin fibroblasts. In rodent cells, oestradiol was shown to cause cell transformation in five studies with different experimental designs, but it gave negative results in two studies. In hamster cells, it gave rise to the formation of DNA adducts, but it did not cause oxidative DNA damage. In human lymphocytes, oestradiol caused micronucleus formation but no chromosomal aberrations or sister chromatid exchange; it weakly induced aneuploidy.

Studies conducted with oestrogens *in vivo* allow the examination of sex- and organ-specific effects. Induction of covalent modifications in DNA was demonstrated by $^{32}P$-postlabelling in the kidneys and liver of hamsters and rats after a subcutaneous implant of oestradiol. In a single study, apparent covalent binding to DNA was not induced in the kidneys or liver of rats or hamsters treated by oral administration with oestradiol; the authors of the study reported that the radiolabel detected in some DNA samples could have been due to protein contamination.

DNA strand breakage was induced in the kidneys but not the livers of hamsters after subcutaneous implantation of oestradiol at low doses and in both liver and kidney after a much higher dose; an even higher dose of oestradiol administered to male hamsters intraperitoneally had no effect on either kidneys or liver.

Oestradiol induced sister chromatid exchange in mouse uterine cells but not in kidney cells *in vivo*, but it did not cause aneuploidy in either cell type. It gave rise to chromosomal aberrations and aneuploidy in renal cells of hamsters exposed *in vivo*.

In a single study, K-, H- or N-*ras* and *p53* gene mutations were not found in endometrial lesions of mice fed a diet containing MNU and oestradiol (Murase *et al.*, 1995).

**Table 14. Genetic and related effects of oestradiol and oestrone and their derivatives and of oestriol**

| Test system | Result[a] | | Dose[b] (LED or HID) | Reference |
|---|---|---|---|---|
| | Without exogenous metabolic system | With exogenous metabolic system | | |
| **Oestradiol** | | | | |
| *Salmonella typhimurium* TA100, TA1535, TA1537, TA1538, TA98, reverse mutation | – | – | 2500 µg/plate | Lang & Redman (1979) |
| *Salmonella typhimurium* TA1535, TA1537, TA1538, reverse mutation | – | – | 500 µg/plate | Ingerowski *et al*. (1981) |
| *Salmonella typhimurium* TA100, TA1535, TA1537, TA1538, TA98, reverse mutation | – | – | 500 µg/plate[c] | Lang & Reimann (1993) |
| DNA strand breaks, cross-links or related damage, mouse brain DNA *in vitro* | (+) | NT | 27.2 | Yamafuji *et al*. (1971) |
| DNA strand breaks, cross-links or related damage, Chinese hamster V79 cells *in vitro* | – | – | 816 | Swenberg (1981) |
| DNA single-strand breaks, rat hepatocytes *in vitro* | – | NT | 82 | Sina *et al*. (1983) |
| DNA repair exclusive of unscheduled DNA synthesis, female C57BL mouse mammary epithelial cells *in vitro* | – | NT | 0.2 | Telang *et al*. (1992) |
| Unscheduled DNA synthesis, Syrian hamster embryo cells *in vitro* | – | NT | 10 | Tsutsui *et al*. ( 1987) |
| Gene mutation, Chinese hamster lung V79 cells, *hprt* locus *in vitro* | – | – | 27.2 | Drevon *et al*. (1981) |
| Gene mutation, Chinese hamster lung V79 cells, ouabain *in vitro* | – | – | 27.2 | Drevon *et al*. (1981) |
| Gene mutation, Syrian hamster embryo cells, *hprt* and $Na^+/K^+$ ATPase loci *in vitro* | – | NT | 10 | Tsutsui *et al*. ( 1987) |
| Sister chromatid exchange, mouse cervical fibroblasts and kidney cells *in vitro* | – | NT | 2.7 | Hillbertz-Nilsson & Forsberg (1985) |
| Sister chromatid exchange, Syrian hamster embryo cells *in vitro* | – | NT | 10 | Tsutsui *et al*. ( 1987) |
| Micronucleus formation, Syrian hamster embryo cells *in vitro* | +[c] | NT | 2.72 | Schnitzler *et al*. (1994) |
| Micronucleus formation, ovine seminal vesicle cells *in vitro* | +[c] | NT | 2.72 | Schnitzler *et al*. (1994) |
| Chromosomal aberrations, Syrian hamster embryo cells *in vitro* | – | NT | 10 | Tsutsui *et al*. ( 1987) |
| Chromosomal aberrations, Syrian hamster embryo cells *in vitro* | – | NT | 8.17 | Tsutsui *et al*. (1997) |

**Table 14 (contd)**

| Test system | Result[a] | | Dose[b] (LED or HID) | Reference |
|---|---|---|---|---|
| | Without exogenous metabolic system | With exogenous metabolic system | | |
| Aneuploidy, male Chinese hamster DON cells *in vitro* | + | NT | 13.6 | Wheeler *et al*. (1986) |
| Aneuploidy, Syrian hamster embryo cells *in vitro* | + | NT | 10 | Tsutsui *et al*. (1987, 1990) |
| Aneuploidy, Syrian hamster embryo cells *in vitro* | + | NT | 0.82 | Tsutsui *et al*. (1997) |
| Cell transformation, BALB/c 3T3 embryo-derived mouse fibroblasts | + | NT | 5.5 | Liehr *et al*. (1987a) |
| Cell transformation, C3H 10T1/2 mouse cells | + | NT | 0.27 | Kennedy & Weichselbaum (1981) |
| Cell transformation, Syrian hamster embryo cells | + | NT | 3 | Tsutsui *et al*. ( 1987) |
| Cell transformation, Syrian hamster embryo cells | NT | + | 3 | Hayashi *et al*. (1996) |
| Cell transformation, Syrian hamster embryo cells | + | NT | 2.72 | Tsutsui *et al*. (1997) |
| Cell transformation, female C57BL mouse mammary epithelial cells | – | NT | 0.2 | Telang *et al*. (1992) |
| Cell transformation, primary baby rat kidney + HPV16 + *ras* | – | NT | 0.27 | Pater *et al*. (1990) |
| Sister chromatid exchange, human lymphocytes *in vitro* | – | NT | 13.6 | Hill & Wolff (1983) |
| Sister chromatid exchange, human lymphocytes *in vitro* | – | – | 27.2 | Banduhn & Obe (1985) |
| Micronucleus formation, human lymphocytes *in vitro* | + | NT | 1.3 | Banduhn & Obe (1985) |
| Chromosomal aberrations, human lymphocytes *in vitro* | – | NT | 100 | Stenchever *et al*. (1969) |
| Chromosomal aberrations, human lymphocytes *in vitro* | – | – | 27.2 | Banduhn & Obe (1985) |
| Aneuploidy, human lymphocytes *in vitro* | (+) | NT | 13.6 | Banduhn & Obe (1985) |
| Aneuploidy, human foreskin JHU-1 fibroblasts *in vitro* | + | NT | 20 | Tsutsui *et al*. (1990) |
| DNA strand breaks, cross-links or related damage, male Syrian hamster kidney and liver *in vivo* | (+) | | 22.5 mg imp × 1, 2 wk | Han & Liehr (1994) |
| DNA strand breaks, cross-links or related damage, male Syrian hamster kidney *in vivo* | + | | 250 µg/d imp × 1, 7 d | Han & Liehr (1994) |
| DNA strand breaks, cross-links or related damage, male Syrian hamster liver *in vivo* | – | | 250 µg/d imp × 1, 7 d | Han & Liehr (1994) |

**Table 14 (contd)**

| Test system | Result[a] | | Dose[b] (LED or HID) | Reference |
|---|---|---|---|---|
| | Without exogenous metabolic system | With exogenous metabolic system | | |
| DNA strand breaks, cross-links or related damage, male Syrian hamster kidney and liver *in vivo* | – | | 150 ip × 1 | Han & Liehr (1994) |
| Sister chromatid exchange, female NMRI mouse uterine cervix and uterine horn epithelial cells *in vivo* | + | | 5 µg sc × 1 | Forsberg (1991) |
| Sister chromatid exchange, female NMRI kidney cells *in vivo* | – | | 5 µg sc × 1 | Forsberg (1991) |
| Chromosomal aberrations, male Syrian hamster renal cortical cells *in vivo* | + | | 125 µg/d imp × 1, 5 mo | Banerjee *et al*. (1994) |
| Aneuploidy, female NMRI mouse uterine cervix and uterine horn epithelial cells *in vivo* | – | | 5 µg sc × 1 | Forsberg (1991) |
| Aneuploidy, female NMRI mouse kidney cells *in vivo* | – | | 5 µg sc × 1 | Forsberg (1991) |
| Aneuploidy, male Syrian hamster renal tubular cells *in vivo* | + | | 20 mg imp × 1, 3.5 mo | Li *et al*. (1993) |
| Increase in nuclear DNA content (aneuploidy), female BALB/c mouse cervicovaginal epithelium *in vivo* | + | | 25 µg sc × 5 | Hajek *et al*. (1993) |
| Binding (covalent) to DNA, male Syrian hamster liver, 8-hydroxy-2′-deoxyguanosine formation *in vitro* | NT | – | 54.5 | Han & Liehr (1995) |
| Binding (covalent) to DNA, Syrian hamster embryo cells *in vitro* | NT | (+) | 1 | Hayashi *et al*. (1996) |
| Binding (covalent) to DNA, female Sprague-Dawley rat liver *in vivo* | – | | 0.3 po × 1 | Caviezel *et al*. (1984) |
| Binding (covalent) to DNA, male and female Syrian hamster kidney and liver *in vivo* | – | | 0.3 po × 1 | Caviezel *et al*. (1984) |
| Binding (covalent) to DNA, male Syrian hamster kidney cortex *in vivo* | + | | 31 mg imp × 1, 2.5 mo | Liehr *et al*. (1986c) |

**Table 14 (contd)**

| Test system | Result[a] | | Dose[b] (LED or HID) | Reference |
|---|---|---|---|---|
| | Without exogenous metabolic system | With exogenous metabolic system | | |
| Binding (covalent) to DNA, male Syrian hamster kidney *in vivo* | + | | 22.5 mg imp × 1, 7 mo | Lu *et al*. (1988) |
| **Oestradiol + testosterone** | | | | |
| Binding (covalent) to DNA, male NBL/Cr rat dorsolateral prostate *in vivo* | + | | NR | Han *et al*. (1995) |
| Binding (covalent) to DNA, male NBL/Cr rat ventral and anterior prostate *in vivo* | – | | NR | Han *et al*. (1995) |
| **17α-Oestradiol** | | | | |
| Chromosomal aberrations, male Syrian hamster renal cortical cells *in vivo* | – | | 105 µg/d imp × 1, 5 mo | Banerjee *et al*. (1994) |
| **4-Hydroxyoestradiol** | | | | |
| Cell transformation, Syrian hamster embryo cells | NT | + | 0.3 | Hayashi *et al*. (1996) |
| DNA strand breaks, cross-links or related damage, male Syrian hamster kidney cells *in vivo* | + | | 250 µg/d imp × 1, 7 d | Han & Liehr (1994) |
| Binding (covalent) to DNA, male Syrian hamster liver, 8-hydroxy-2′-deoxyguanosine formation *in vitro* | NT | + | 57.9 | Han & Liehr (1995) |
| Binding (covalent) to DNA, Syrian hamster embryo cells *in vitro* | NT | + | 1 | Hayashi *et al*. (1996) |
| Binding (covalent) to calf thymus DNA *in vitro* | NT | +[d] | 66.7 | Cavalieri *et al*. (1997) |
| Binding (covalent) to DNA, female Sprague-Dawley rat mammary cells *in vivo* | + | | 250 µg i-mam × 1 | Cavalieri *et al*. (1997) |

**Table 14 (contd)**

| Test system | Result[a] | | Dose[b] (LED or HID) | Reference |
|---|---|---|---|---|
| | Without exogenous metabolic system | With exogenous metabolic system | | |
| **2-Hydroxyoestradiol** | | | | |
| Cell transformation, Syrian hamster embryo cells | NT | + | 1 | Hayashi *et al*. (1996) |
| DNA strand breaks, cross-links or related damage, male Syrian hamster kidney cells *in vivo* | - | | 250 µg/d imp × 1, 7 d | Han & Liehr (1994) |
| Binding (covalent) to DNA, male Syrian hamster liver, 8-hydroxy-2′-deoxyguanosine formation *in vitro* | NT | – | 57.9 | Han & Liehr (1995) |
| Binding (covalent) to DNA, Syrian hamster embryo cells *in vitro* | NT | + | 1 | Hayashi *et al*. (1996) |
| **Oestradiol-3,4-quinone** | | | | |
| Binding (covalent) to calf thymus DNA *in vitro* | + | NT | 200 | Cavalieri *et al*. (1997) |
| Binding (covalent) to DNA, female Sprague-Dawley rat mammary cells *in vivo* | + | | 250 µg i-mam × 1 | Cavalieri *et al*. (1997) |
| **Oestrone** | | | | |
| Gene mutation, Chinese hamster lung V79 cells, *hprt* locus *in vitro* | – | – | 27 | Drevon *et al*. (1981) |
| Gene mutation, Chinese hamster lung V79 cells, ouabain *in vitro* | – | – | 27 | Drevon *et al*. (1981) |
| Binding (covalent) to DNA, male Syrian hamster liver, 8-hydroxy-2′-deoxyguanosine formation *in vitro* | NT | – | 54.1 | Han & Liehr (1995) |
| Binding (covalent) to DNA, female Sprague-Dawley rat liver *in vivo* | – | | 0.3 po × 1 | Caviezel *et al*. (1984) |
| Binding (covalent) to DNA, male Syrian hamster kidney and liver *in vivo* | – | | 0.3 po × 1 | Caviezel *et al*. (1984) |
| **Oestrone-3,4 quinone** | | | | |
| DNA strand breaks, cross-links or related damage, human MCF-7 cells *in vitro* | + | NT | 7.1 | Nutter *et al*. (1994) |
| Binding (covalent) to calf thymus DNA *in vitro* | (+) | NT | 200 | Cavalieri *et al*. (1997) |

**Table 14 (contd)**

| Test system | Result[a] | | Dose[b] (LED or HID) | Reference |
|---|---|---|---|---|
| | Without exogenous metabolic system | With exogenous metabolic system | | |
| **16α-Hydroxyoestrone** | | | | |
| DNA repair exclusive of unscheduled DNA synthesis, female C57BL mouse mammary epithelial cells *in vitro* | + | NT | 0.2 | Telang *et al*. (1992) |
| Cell transformation, female C57BL mouse mammary epithelial cells | + | NT | 0.2 | Telang *et al*. (1992) |
| **2-Hydroxyoestrone** | | | | |
| Binding (covalent) to DNA, male Syrian hamster liver, 8-hydroxy-2′-deoxyguanosine formation *in vitro* | NT | – | 57.5 | Han & Liehr (1995) |
| **4-Hydroxyoestrone** | | | | |
| Binding (covalent) to calf thymus DNA *in vitro* | NT | +[d] | 66.7 | Cavalieri *et al.* (1997) |
| Binding (covalent) to DNA, male Syrian hamster liver, 8-hydroxy-2′-deoxyguanosine formation *in vitro* | NT | + | 57.5 | Han & Liehr (1995) |
| **Oestriol** | | | | |
| DNA repair exclusive of unscheduled DNA synthesis, female C57BL mouse mammary epithelial cells *in vitro* | – | NT | 0.2 | Telang *et al*. (1992) |
| Aneuploidy, male Chinese hamster DON cells *in vitro* | + | NT | 21.6 | Wheeler *et al*. (1986) |
| Cell transformation, female C57BL mouse mammary epithelial cells | – | NT | 0.2 | Telang *et al*. (1992) |
| Sister chromatid exchange, human lymphocytes *in vitro* | (+) | NT | 14 | Hill & Wolff (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; wk, week; d, day; mo, months; imp, subcutaneous implant; ip, intraperitoneal injection; sc, subcutaneous injection; po, oral; i-mam, intramammary; NR, not reported

[c] 41–68% of the induced micronuclei contained CREST-reactive kinetochores

[d] Horeradish peroxidase-activated or lactoperoxidase-activated or S9 activated system

A combination of oestradiol and testosterone administered to male rats resulted in DNA binding in the dorsolateral prostate but not in the ventral or anterior prostate.

In a single study, the frequency of chromosomal aberrations was not increased in renal proximal convoluted tubules of male hamsters treated with 17α-oestradiol.

DNA strand breakage was demonstrated in kidney cells of male hamsters treated subcutaneously with 4-hydroxyoestradiol. In hamster cells *in vitro*, 4-hydroxyoestradiol caused cell transformation and formation of DNA adducts in the presence of exogenous metabolic activation. Induction of oxidative damage in male hamster liver DNA and binding to calf thymus DNA were seen after in-vitro treatment with 4-hydroxyoestradiol, and similar results were observed *in vivo* in mammary cells of rats treated with this compound.

DNA strand breakage was not demonstrated in kidney cells from male hamsters treated subcutaneously with 2-hydroxyoestradiol, and this compound did not bind to liver DNA of hamsters *in vitro* in one study. In hamster cells, 2-hydroxyoestradiol caused cell transformation and formation of DNA adducts in the presence of exogenous metabolic activation.

Oestradiol-3,4-quinone bound to DNA both *in vitro* and *in vivo* in rat mammary cells.

Oestrone did not cause gene mutation at various loci in hamster ovary cells. It did not induce oxidative damage in hamster liver DNA, nor did it bind to kidney or liver DNA of male hamsters or to liver DNA of rats treated *in vivo*.

*In vitro*, oestrone-3,4-quinone induced DNA strand breaks in human MCF-7 cells and bound weakly to calf thymus DNA.

In a mammary cell line derived from mice, DNA repair and cell transformation were induced by treatment with 16α-hydroxyoestrone.

No induction of oxidative DNA damage was seen in the presence of an exogenous metabolic activation system in male hamster liver cells treated *in vitro* with 2-hydroxyoestrone, but 4-hydroxyoestrone was active in this assay. Furthermore, the latter compound bound to calf thymus DNA under these conditions.

Neither DNA repair nor cell transformation was induced in mouse mammary epithelial cells treated with oestriol, whereas aneuploidy was induced in male hamster DON cells. Oestriol weakly induced sister chromatid exchange in human lymphocytes *in vitro*.

In one study, equilin and equilenin decreased the formation of single- and double-strand DNA breaks induced by hydrogen peroxide alone or with $Cu^{2+}$ (Tang & Subbiah, 1996).