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Review

The effects of environmental hormones on reproduction

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Abstract. Considerable attention has been given in the past few years to the possibility that man-made chemicals (xenobiotics) in the environment may pose a hazard to human reproductive health. The endocrine-disrupting effects of many xenobiotics can be interpreted as interference with the normal regulation of reproductive processes by steroid hormones. Evidence reviewed here indicates that xenobiotics bind to androgen and oestrogen receptors in target tissues, and to androgen-binding protein and to sex hormone-binding globulin. Although environmental chemicals have weak hormonal activity, their ability to interact with more than one steroid-sensitive pathway provides a mechanism by which their hazardous nature can be augmented. A given toxicant may be present in low concentration in the environment and, therefore, harmless. However, we are not exposed to one toxicant at a time, but, rather, to all of the xenobiotics present in the environment. Therefore, numerous potential agonists/antagonists working together through several steroid-dependent signalling pathways could prove to be hazardous to human reproductive health.

Key words. Xenobiotics; reproduction; steroid hormones; steroid receptors; androgen-binding protein; sex hormone-binding globulin; environmental toxicants.

Introduction

During the past 50 or more years, vast quantities of diverse synthetic chemicals (xenobiotics) have been released into the environment as a consequence of efforts expended to increase agricultural productivity and as a result of modern manufacturing processes and their by-products. These chemicals include herbicides, pesticides, fungicides, plasticizers, polystyrenes, polychlorinated biphenyls (PCBs), polychlorinated dibenzodioxins and alkylphenolic compounds [1–3]. We have recently become aware of the fact that some environmental xenobiotics can interfere with the normal embryonic development of the male and female reproductive systems of wildlife and experimental animals and that they can disrupt normal reproductive function in adulthood [1, 3, 4]. Although direct evidence is lacking, theoretical considerations and epidemiological evidence implicate these compounds as potential hazards to human reproductive health [4–11].

The original concept that environmental pollutants might pose a threat to reproduction was not based on theory, but rather was derived from the observations of wildlife biologists in the field. They noted that the population of colonial fish-eating birds in the Great Lakes basin of the United States was declining [12–14]. This area is highly contaminated with various organo-

chlorine compounds, including dioxins, PCBs and 1,1,1 trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) congeners [14]. The reproductive problems that these early workers recorded were of a generalized nature including eggshell breakage, poor hatchability and abnormal parental behaviour [14]. Gilbertson et al. [14] reviewed the extensive literature to ascertain if the data supported the hypothesis that reproductive impairment in the birds was a result of exposure to pollutants in the contaminated waters. They concluded that the evidence cohered with the interpretation that the outbreak of the syndrome of reproductive defects was caused by the presence of pollutants in the areas where the birds fed. Many other investigators have obtained additional supporting data by observing reproductive dysfunction in numerous species inhabiting areas contaminated with environmental toxicants [1, 3, 4, 15]. The data indicate that fish, birds, reptiles, mammals and other species inhabiting environments polluted with a number of known and unknown synthetic compounds suffer reproductive problems. Several general reviews of this literature have been published [1, 3, 4, 15].

The observation that many of the reproductive alterations seen in wildlife species inhabiting contaminated environments resemble those one might anticipate from oestrogen agonists or antagonists has given rise to the concept that the environmental toxicants brought about their effects by acting as oestrogen mimetics, xenooestrogens [1, 4, 9, 10]. The facts that plants contain some compounds that exhibit oestrogenic activity [16–18], and that these compounds are able to interact with the oestrogen receptor [18] and alter reproductive function [19, 20], have reinforced this concept and have led investigators to the conclusion the phytooestrogens present in foodstuffs are also potential hazards to reproductive health, especially in humans [9, 10]. Recent reports indicate that certain xenobiotics can act as androgen antagonists/agonists [21–26] and that several common environmental pollutants preferentially inhibit the binding of radiolabelled androgens to the androgen receptor [27]. In light of these findings it is necessary to expand the concept of oestrogen mimetics to a concept of environmental hormones. The observations that chemicals in the environment can interfere with the physiological endocrine-regulated processes that control the development and function of male and female reproductive systems has led to the coining of the term 'endocrine disruptors' to describe the mechanism of action of these compounds [1, 4]. This review will concentrate on those endocrine disruptors that are likely to interfere with reproductive function by altering the steroid hormone-dependent pathways that regulate these processes. PCBs and dioxin have been implicated in impairing thyroid function [28–30]. Thyroid hormone acts through its nuclear receptor, which is a member of the steroid/thyroid hormone family of ligand-dependent transcription factors [31]. Whether the adverse effects attributed to the toxicants are mediated through the thyroid hormone receptor is not clear. Dietary flavanoids inhibit thyroid peroxidase, the enzyme that catalyses thyroid hormone synthesis [32, 33]; this could obviously lead to a diminution of thyroid function. The topic of disruption of thyroid function by environmental toxicants will not be discussed further in this review. Other mechanisms for endocrine disruption would include action through the aryl hydrocarbon (Ah) receptor [34], interference with steroid or cellular metabolism [35–39], or action through other non-genomic mechanisms [40].

Evidence supporting the hypothesis that environmental xenobiotics are endocrine disruptors

Most of the references cited above providing evidence that reproductive function of animals living in their natural habitats can be compromised by exposure to environmental toxicants deal with observational and/or epidemiological data. However, an increasing number of experimental studies reinforce this concept.

Evidence for the interaction of xenobiotics with steroid receptors

As long as 30 years ago, laboratory studies showed that DDT analogues had oestrogenic effects on the mammalian uterus, on the avian oviduct and on other tissues [41–43]. These compounds also inhibit oestradiol binding to the oestrogen receptor [42, 43]. Nelson determined that the most potent inhibitor was 1,1,1-trichloro-2-(*p*-chlorophenyl)-2-(*o*-chlorophenyl)-ethane (*o*,*p*′-DDT). It produced 50% inhibition of oestradiol binding to its receptor at a concentration that was 2000 times greater than that required of the powerful oestradiol agonist diethylstilboestrol (DES). The other polychlorinated hydrocarbons tested [43] were orders of magnitude less effective than *o*,*p*′-DDT, or they did not inhibit oestradiol binding to the receptor. The inhibition of [^{3}H]oestradiol binding caused by *o*,*p*′-DDT appeared to be competitive, and in vivo studies showed a positive correlation between the ability of the compounds to increase uterine weight and their relative affinity for the receptor [42, 43].

Chlordecone (Kepone), a chlorinated hydrocarbon insecticide, was shown to be oestrogenic in the rat uterus and to interact with the uterine oestrogen receptor [44]. Polychlorinated hydroxybiphenyls also inhibit oestradiol binding to its receptor [45]. Methoxychlor, the bis-*p*-methoxy derivative of DDT, did not inhibit oestradiol binding to rat uterine cytosol, but its dimethylated derivative did [46]. Alkylphenols are able

to prevent the binding of oestradiol to the uterine oestrogen receptor and to displace oestradiol that is already bound [47, 48].

In addition to these studies showing that synthetic compounds can interact with the oestrogen receptor, similar observations were made concerning phytooestrogens [49]. Furthermore, experimental studies have shown that plant oestrogens affect animal reproduction [19] and sexual differentiation [20], and have generalized oestrogenic effects in an oestrogen-dependent cell line and in vivo [18].

Until recently [21–25, 27] there appears to have been only one study that examined the possibility that chemicals in the environment might interact with the androgen receptor [49]. This study was prompted by an epidemic of gynaecomastia in Haitian men and the suggestion that a pyrethroid-containing insecticide to which they were exposed had antiandrogenic properties. The investigators determined that several pyrethroids were able to inhibit competitively the binding of a synthetic radiolabelled androgen to the androgen receptor [49].

The above survey indicates that as early as the mid-1970s experimental data were available that should have provided a warning that certain environmental compounds could act as oestrogen or androgen agonists/antagonists. The data also provided a clue as to the molecular mechanisms by which xenobiotics could act, namely by interfering with the steroid hormone receptor-mediated pathways that regulate the development and function of the reproductive system. Some of these data were obtained fully 15 years prior to acute awareness of pollutant-mediated reproductive dysfunctions in wildlife species [1, 4].

Experimental evidence of xenobiotic-induced effects on animal reproduction

As described above, *o*,*p*′-DDT competitively inhibits the binding of [^{3}H]oestradiol to the oestrogen receptor [42, 43]. Further studies showed that *o*,*p*′-DDT administered to immature female rats stimulated DNA synthesis and cell division in uterine epithelial and stromal cells in a manner similar to that caused by oestradiol administration [50]. It also produces uterine hyperplasia, a characteristic oestrogenic response. Of particular interest is the observation that retention of the *o*,*p*′-DDT-receptor complex in uterine nuclei was more prolonged than the retention of the oestradiol-receptor complex. This finding may have broad implications concerning how environmental chemicals could interact with the steroid receptor pathway but bring about effects different from those caused by endogenous hormones.

The persistent DDT metabolite, *p*,*p*′-DDE, has been shown to inhibit the binding of [^{3}H]R1881, a synthetic androgen, to the androgen receptor present in rat prostate cytosol, to prevent androgen-induced transcriptional activity, and to result in abnormalities of male sexual development [22].

Treatment of male rats with methoxychlor reduced the weight of the seminal vesicles, cauda epididymis and pituitary. It also decreased the sperm content of the epididymis and, at 100 and 200 mg/kg doses, caused a delay in the attainment of puberty. Despite these reproductive changes, the males were fertile [51]. In females, the age of vaginal opening and first oestrus were delayed, and reproductive tract anomalies were noted. Females receiving the highest dose of methoxychlor went from constant oestrus into pseudopregnancy following mating, but no implantation sites were observed upon necropsy [51].

The fungicide vinclozolin [3-(3,5-dichlorophenyl)-5-vinyl-oxazoladine-2,4-dione] has been shown to disrupt sexual differentiation of male rats [23]. In this study, pregnant rats were treated beginning on gestational day 14, when the embryos are at the ambisexual stage of development. The anogenital distance in the males was female-like at birth, and nipple development was prominent at 2 weeks of age. Many of the males had ectopic testes, a vaginal pouch, epididymal granulomas and small to absent accessory sex organs. All males had a cleft phallus with hypospadias. The data on the failure of prostate development and the ectopic location of the testes are similar to those obtained following treatment of rats with the antiandrogen flutamide [21, 52], suggesting that vinclozolin acts as an androgen receptor antagonist. This concept is reinforced by data showing that vinclozolin does not inhibit the enzyme 5α-reductase, but that it, and especially some of its in vivo metabolites, can bind to the androgen receptor [21]. It is likely that the antiandrogenic effects of vinclozolin are attributable to its metabolites rather than to the parent compound [26]. In the absence of the androgen 5α-dihydrotestosterone (5α-DHT), a vinclozolin metabolite promotes binding of the androgen receptor to its DNA response element and activates androgen-dependent transcription [25]. These data indicate that vinclozolin metabolites can act as an androgen agonists or antagonists depending on the hormonal context.

Another synthetic compound in the environment that has been shown to have adverse effects on male reproduction is the fungicide methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate (benomyl). This compound causes a reduction in spermatocyte number and produces multinucleated germ cells [53]. It also causes sloughing of germ cells, seminiferous tubule atrophy, occlusion of the efferent ducts and other abnormalities of the male reproductive system [53]. Since the efferent ducts contain oestrogen receptors [54–56] and fluid resorption by the ducts appears to be under oestro-

genic control [57], it is possible that the effects of benomyl, at least on these structures, is oestrogenic or anti-oestrogenic.

PCBs are manufactured through the progressive chlorination of biphenyl, theoretically, giving rise to 209 congeners [4, 11]. PCBs have been used extensively for various industrial purposes, including constituents of insecticides and as an insulating material [4]. Although PCBs have been banned from use in industrialized countries since the 1970s [4], they are one of the most persistent and widespread of the xenobiotics in the ecosystem because of their chemical stability and their ability to bioaccumulate [1, 3, 58, 59]. PCBs have been shown to act through the Ah receptor to bring about their biological effects [34]; however, new evidence indicates that they also act through the oestrogen receptor [60]. These investigators examined a specific PCB congener, 3,4,3′,4′-tetrachlorobiphenyl (TCB) in their studies. They showed that TCB could competitively inhibit binding of [^{3}H]oestradiol to cytosol prepared from oestrogen-responsive MCF-7 breast cancer cells, that it induced oestrogen receptor binding to DNA, that it was able to transactivate an oestrogen-responsive reporter gene, that it stimulated growth of the cells and that it acted in vivo to increase the weight of the mouse uterus [60]. These activities can be considered hallmarks of physiological oestrogens. PCBs have been shown be to antiandrogenic in the cockerel [61], and various oestrogenic and antioestrogenic effects of PCBs have been reported [62–65]. Thus, it would appear that the potential exists for various PCB congeners to cause endocrine-disruptive effects by acting as oestrogen receptor agonists/antagonists or as androgen receptor antagonists.

The herbicide Linuron [3-(3,4-dichlorophenyl)-1-methoxy-1-methyl-urea] also has been shown to be a disruptor of male reproductive tract development [66]. Linuron treatment of rats resulted in a statistically significant reduction in the weight of the epididymis and accessory sex organs in sexually immature animals; it caused a reduction in weight of only the accessory organ unit and prostate in an adult group. Increased serum levels of oestradiol and luteinizing hormone (LH) were observed in the sexually mature rats treated with Linuron. The alterations caused by Linuron were similar to those produced by the antiandrogen flutamide [52, 66]. Linuron was also shown to compete with [^{3}H] testosterone for binding to the prostate androgen receptor [66]. Taken together, these data are consistent with the hypothesis that Linuron brings about its effects in the male by acting as an antiandrogen.

The hexachlorocyclohexanes (HCHs) are mixtures of stereoisomers differing in the relative positions of chlorine around the boat and chair forms of the hexane ring [59]. The γ-isomer (HCHγ) is sold as the commercial insecticide, Lindane. Chronic peroral treatment of weanling female rats with HCHγ delayed vaginal opening and disrupted ovarian cyclicity [67]. It has also been shown to accumulate in reproductive tissues of female rabbits [68]. In a recent study [69], it was shown that Lindane does not alter the affinity or the concentration of the oestrogen receptor or the oestrogen-dependent induction of the progesterone receptor in immature or ovariectomized adult female rats. Other investigators have shown that Lindane binds to the androgen receptor present in the rat prostate [70] and causes biochemical and histological changes in the rat testis [71]. We [27] have shown that the δ isomer of HCH (HCHδ) binds to the androgen receptor, to androgen-binding protein (ABP) and to sex hormone-binding globulin (SHBG) [27].

Cyclodiene insecticides, such as dieldrin, have been shown to have detrimental effects on reproduction and fertility in several species [59]. Compounds of this class have been shown to decrease oestrogen-sensitive reporter activity in a yeast system [72], providing evidence for a possible antioestrogenic activity. It has recently been shown that dieldrin inhibits [^{3}H]5α-DHT binding to the androgen receptor [27].

Reports indicate that the plasticizer di(2-dihexyl)-pthalate has adverse effects on testicular morphology [73]. The fact that pthalates are weak oestrogens [74] may explain these effects.

Alkylphenolic compounds are nonionic surfactants that are widely used in detergents, cosmetics, paints, herbicides, pesticides and other products [1, 4]. Soto et al. have shown that an alkylphenol, nonylphenol, which is released from plastic centrifuge tubes, is oestrogenic [75]. Recently White et al. [47] showed that octylphenol, nonylphenol and two alkylphenol polyethoxylates were able to stimulate oestrogen-dependent vitellogenin gene expression in trout hepatocytes, oestrogen-dependent gene transcription in transfected cells and growth of breast cancer cell lines. Octylphenol was the most potent of these chemicals and was able to stimulate these processes to the same extent as oestradiol; however, a 1000-fold greater concentration was required [47]. The interaction of alkylphenols with the oestrogen receptor has been demonstrated [48]. Nonylphenol appears to be a competitive inhibitor of oestradiol binding to the catfish hepatic oestrogen receptor and to stimulate vitellogenin synthesis above control levels. However, the stimulation was far less than that which occurred in the presence of oestradiol [76]. Nonylphenol inhibits oestrogen binding to the oestrogen receptor and inhibits androgen binding to ABP and SHBG [27].

Recent studies indicate that alkylphenols have detrimental effects on male reproductive parameters. In one study, octylphenol or benzylphthalate was administered to male rats during gestation or during the first 21 days of postnatal life [77]. Reproductive parameters were

then evaluated during adulthood. A small but statistically significant reduction in mean testicular size was noted. Octylphenol reduced ventral prostate weight. Both octylphenol and benzylphthalate caused a statistically significant reduction in daily sperm production [77]. Another study examined the effects of octylphenol on the secretion of reproductive hormones in adult male rats [78]. The data demonstrate that chronic administration adversely affected the secretion of LH, follicle stimulating hormone (FSH), prolactin and testosterone, and that these effects mirrored those caused by oestrogens. Octylphenol administration decreased the weight and altered the histological features of the testis, epididymis and accessory sex organs, and caused an increase in the proportion of abnormal sperm [79]. These alterations of male reproductive function caused by treatment of rats with alkylphenols were presumed by the investigators to be oestrogenic effects of the compounds. An oestrogen-inducible strain of yeast expressing the human oestrogen receptor was used to determine the structural features of alkylphenolic compounds that are responsible for their oestrogenic activity [80]. It was concluded that the optimal oestrogenic activity requires a single tertiary branched alkyl group composed of six to eight carbons located at the para position of an otherwise unhindered phenol ring [80] (fig. 1).

Bisphenol-A (BPA), a monomer of polycarbonate plastics, has also been shown to have oestrogenic properties [81–83]. This compound was shown to compete with [^{3}H]oestradiol for binding sites on the oestrogen receptor [81]. BPA was shown to induce the prolactin gene, but at a 1000–5000-fold lower potency than oestradiol [82]. Interestingly, BPA could induce hyperprolactinaemia in oestrogen-sensitive rats when administered in vivo at an efficiency similar to that of oestradiol [82]. It was reported recently [84] that BPA induces morphological and molecular changes in the rat uterus and vagina that are similar to those caused by oestradiol. Structure/function studies indicate that the oestrogenicity of bisphenols is influenced by the length and nature of the alkyl substituents attached to the carbon that bridges the two phenolic rings that constitute the bisphenol molecules [85].

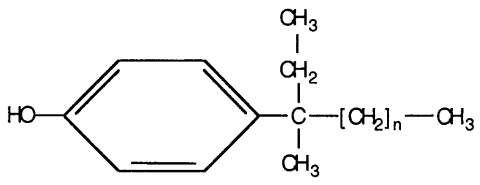

Figure 1. Model of an alkylphenolic compound exhibiting the structural requirements for maximal oestrogenic activity.

Chlorophenols are herbicides and antimicrobial agents used in the preservation of wood [59]. Pentachlorophenol has been used as an agricultural fungicide. It has also been used in food-processing plants to control mould and slime and in industry for processing textiles and cellulose materials [59]. Polychlorinated benzo-*p*-dioxins have been found to contaminate many commercial pentachlorophenol preparations [59]. The dioxins are known to have many effects on reproduction, especially in males [86–88]. The effects of dioxins in these processes are probably mediated through the Ah receptor [34], not directly through steroid receptors. However, the striking antioestrogenic activity of these compounds may be mediated by cross-talk between the Ah receptor and oestrogen-mediated signalling [89]. Dioxins will not be discussed further here since this area is adequately reviewed [34].

It is of considerable interest, and it may be of predictive value, that many phenolic compounds have oestrogenic activity. Early studies postulated that phenolic metabolites of DDT might account for their oestrogenic activity [42]; however, this has not been demonstrated conclusively [42, 43]. A phenolic compound, bis-(4-hydroxyphenol) [2 - (phenoxysulphonyl)phenyl] methane, isolated from the phenol red indicator used in tissue culture media [90], has been shown to be weakly oestrogenic [91]. Other oestrogenic phenols include the PCBs, alkylphenols and BPA. Although some of these compounds bear a structural resemblance to DES [41], others do not appear to. What is needed are computer models that can predict the three-dimensional configuration of these and other xenobiotics so that their ability to fit into the binding site of steroid receptors can be determined. Attempts at such modelling have been done for potential oestrogen and androgen receptor agonists/antagonists [92, 93].

In addition to the compounds discussed above, over 40 other chemicals that are widely distributed in the environment have been reported to have effects on reproduction or to have other endocrine-disrupting effects [1, 4]. However, this number must be considered incomplete, since literally thousands of the man-made chemicals in the environment and most of the phytocompounds have yet to be evaluated for their endo-crine-disrupting effects or for their effects on the development and function of reproductive systems.

There is no direct evidence for pollutant-induced reproductive disorders in humans

There is no direct evidence that environmental reproductive toxicants have deleterious effects in humans. However, the clinical use of the potent synthetic oestrogen DES provides human data that can be compared to those obtained in experimental systems. Clinical studies have demonstrated that DES, which was administered

to women with a history of spontaneous abortions, results in abnormalities of the reproductive tract of a statistically significant number of male and female offspring [4, 94]. Many of the deleterious effects seen in the offspring of DES-treated mothers resemble the reproductive tract abnormalities discussed above in animals exposed to environmental xenobiotics under natural and experimental conditions [95]. Thus far, DES is the only man-made chemical that has been shown to have deleterious effects on the development and/or function of the human reproductive tract.

Studies on experimental animals using DES or other oestrogens in the prenatal or neonatal period have produced effects that are similar to those obtained in humans, such as atypical epithelium in the vagina and cervix [96]. Serious and lasting disturbances of pituitary-hypothalamic function reflected in loss of cycling and a state of constant vaginal cornification have been observed [97]. Administration of oestrogens to developing male animals results in many reproductive tract lesions, including disruption of testicular function, cryptorchidism, and hypertrophy and squamous metaplasia of accessory sex organs [97–101]. Oestradiol administration causes a dose-dependent suppression of blood levels of FSH resulting in the reduction of testis weight and sperm production [101]. Of particular interest is a report that neonatal treatment of hamsters with DES disrupts reproductive function in the adult after apparently normal pubertal development [102]. Numerous lesions were observed in these animals, including involution of the epididymal epithelium, abnormal seminal vesicle morphology and disrupted seminiferous tubules with absence of developing germ cells. All of these changes occurred despite normal circulating testosterone levels [102].

What evidence exists that environmental toxicants affect human reproductive health? The only evidence is circumstantial, but it is compelling. Chilvers et al. [5] showed that there was a doubling of the frequency of undescended testes in England and Wales between 1962 and 1981. A more recent study, showed that the incidence of cryptorchidism had increased by 65% over the past 2 decades [6]. Hypospadias in boys has more than doubled over the last 40 years [7]. Moreover, there is convincing, though controversial, evidence that semen quality has changed over the past 50 years [8]. Although this report has been questioned [103], the authors of a review that examines this issue extensively appear to support the conclusion that the decline in sperm count is a real phenomenon [4]. One of the most adverse trends in male reproductive health during the past few decades has been an increase in the incidence of testicular cancer in several countries [4]. Furthermore, a recent paper reviews evidence that the ratio of male to female births is declining in several industrialized countries [104]; the authors speculate that environmental factors may be involved in the decline. Sharpe and Skakkebaek [9] and Sharpe [10] have postulated that many disorders in male reproductive function arise from in utero exposure of male embryos to environmental oestrogens. However, in light of the data indicating that some xenobiotics are androgen agonists/antagonists [21–23, 25], one cannot exclude the possibility that some of the oestrogen-like deficits seen may actually be the result of androgenic/antiandrogenic actions of environmental pollutants. Clinical reproductive problems that may arise from exposure to environmental toxicants have been reviewed extensively [4].

Evidence that the environment is contaminated with known endocrine disruptors

Although many xenobiotics in the environment have weak hormonal activity as compared with endogenous steroids [1, 4, 82], their lipophilic nature and long half-lives allow them to accumulate and persist in fatty tissues of the body, thus increasing their concentration and bioavailability [14, 105–107]. Many studies have been conducted to determine the concentration of environmental contaminants, especially organochlorine compounds, in the tissues of various species [108, 109] including humans [110]. Due to its high lipid content, organochlorine chemicals accumulate in breast milk [111, 112]. Therefore, infants are likely to be exposed to higher concentrations of xenobiotics during nursing than at any other time.

Another route of exposure of infants to potential reproductive toxicants is through being fed soy-based infant formulas. Evidence is available indicating that if infants were fed such formulas exclusively, the result would be a mean daily intake of phytooestrogenic isoflavones of 20 mg/kg body mass [113]. This dose is about 25 times higher than the dose that was shown to slightly prolong the menstrual cycle in women [114]. Such formulas lead to circulating concentrations of isoflavones 13,000–22,000 times higher than circulating oestradiol levels [115]. Since the levels of endogenous oestrogens in male and female infants are low, extended exposure to high levels of even weakly oestrogenic phytooestrogens might pose serious problems to their reproductive systems. Cow's milk also can be a source of exposure to exogenous oestrogens [4].

The accumulation of endocrine-disrupting xenobiotics in food sources, such as fish and animal meat, offers the potential of exposing the human population to increased concentrations of the compounds. It was the endocrine-disrupting effects of accumulated xenobiotics in fish that birds ate that provided the first evidence for

the harmful effects of these compounds on reproduction (see above). Endocrine-disrupting pollutants are also found in the water we drink [116]. Cans in which foods are preserved are lined with BPA [83]. Organochlorine pollutants are found in the oceans, and they can accumulate in sea mammals [117]. The persistence and worldwide distribution of hazardous organochlorine compounds caused by global distillation has been documented [118]. Organochlorine insecticide residues have been shown to be present even in the tissues of remote African fauna [119]. Thus, human exposure to potential disruptors of reproductive function is widespread.

Even though it appears that xenobiotics have hormonal effects at concentrations that are orders of magnitude higher that required for physiological hormones, our wide exposure to large numbers of different environmental man-made compounds is a cause for concern. McLachlan's group [120] presented data showing synergistic activation of the oestrogen receptor by combinations of environmental xenobiotics. Such a process would have helped to explain how even low concentrations of individual xenooestrogens might bring about harmful effects. However, the mechanisms by which this synergism could occur in a manner that would be consistent with what is known concerning the structure of steroid hormone receptors [121] is not obvious. This is particularly so since Scatchard [122] analysis of their binding data resulted in linear slopes, indicating that oestradiol and the xenobiotics were interacting with the same, or an equivalent, site on the receptor. The validity of the data presented by McLachlan was questioned by others who were unable to duplicate the published results [123]. Subsequently, the original paper was withdrawn by the authors [124]. Our data [27] indicating that xenobiotics may affect multiple signalling pathways by interacting with more than one steroid receptor or binding protein may help to provide an alternative mechanism by which the detrimental effects of environmental toxicants can be amplified.

Sexual differentiation and development

Vertebrate embryos initially exhibit an ambisexual stage during which an indifferent gonad is present. This gonad can differentiate into either an ovary or testis. Also present in the embryo are Müllerian and Wolffian ducts. The former structures are the anlage of the oviducts, uterus and upper part of the vagina. The Wolffian ducts give rise to the epididymis, vas deferens and seminal vesicles [125]. Under the influence of the *SRY* gene on the Y chromosome and of several downstream effectors and autosomal genes (e.g. *SOX9* and *SF-1*), the indifferent gonad develops into a testis [126–129]. The Sertoli cells of the testis secrete Müllerian inhibiting substance (MIS), which causes the regression of the Müllerian ducts [130–132]. Testosterone secretion by the Leydig cells of the developing testis induces the differentiation of the Wolffian ducts into the internal structures of the male reproductive tract; the masculinization of the external genitalia requires the conversion of testosterone to 5α-DHT [52, 133]. The female reproductive system develops in the absence of the SRY gene and, consequently, in the absence of MIS and androgens. The female pathway is considered to be independent of hormonal regulation [130]. Nonetheless, the foetal rabbit ovary has been shown to produce oestradiol at the same age as the testis was able to synthesize testosterone [134]. Oestrogen receptors are present in the mouse embryo at the ambisexual stage [135], and they are also present in early embryos of other species [136, 137]. Therefore, it is likely that oestrogens are involved in the differentiation of structures derived from the Müllerian ducts, and they may affect differentiation of Wolffian derivatives. Sexual differentiation in humans occurs during weeks 7–12 of gestation [125].

Several windows, then, exist during the differentiation of the male and female reproductive systems during which environmental steroid agonists/antagonists could interfere with their physiological development. During the critical period of sexual differentiation, one might anticipate that exposure of a chromosomal male to antiandrogenic xenobiotics would interfere with the androgen-dependent differentiation of the Wolffian-derived structures and/or with the normal development of the male genitalia. Since oestrogen receptors are present in the male reproductive tract [54, 55, 57, 135, 137–140], xenooestrogens could also interfere with the proper development of these structures. Likewise, xenoandrogens could masculinize the developing female foetuses, since the structures of their reproductive tracts contain androgen receptors [141]. The genital tract in males and females is laid down during embryogenesis, but it is not fully differentiated until acted upon by rising levels of sex hormones that occur during puberty [142]. The initiation of the functional activation of the male and female reproductive systems provides another occasion during which environmental endocrine disruptors could act to alter normal physiology. Therefore, one would anticipate that the greatest risks to reproductive health posed by xenobiotics would be during the embryonic, neonatal and pubertal periods, when the reproductive systems are undergoing finely tuned modulation by steroid hormones. The endocrine-disrupting effects that occur in utero often do not manifest themselves until puberty or even later [94, 96, 98]. Therefore, to fully understand the potential deleterious effects of xenobiotics on reproduction, studies must be conducted over the life span of experimental animals, and careful long-range epidemiological studies must be done in humans.

Since the reproductive tissues of both sexes contain androgen and oestrogen receptors [143], the molecular basis for direct effects of androgens (xenoandrogens) and oestrogens (xenooestrogens) exists in males and females. That oestrogens have effects on male reproductive tissues has been known for some decades [144]. The development of a mutant mouse model that lacks responsiveness to oestradiol because of insertional disruption of the oestrogen receptor gene by gene targeting [the oestrogen receptor knockout (ERKO) mouse] [145] and the expectation that so-called environmental oestrogens might have deleterious effects in the male [9, 10] have renewed interest in oestrogen action in the male. Recent papers on oestrogen action in the male [54, 55, 135] tend to neglect the extensive older literature which reported such effects as histological alterations of the reproductive tract [98, 144] and effects on protein synthesis [146, 147]. A recent paper by Hess et al. [57] using the ERKO mouse model [145] determined that oestradiol regulates the absorption of fluid by the efferent ducts of the testis, thus providing direct evidence for a specific, physiological role for oestrogens in the male.

Neither the paper by Hess et al. [57] nor the paper by Kuiper et al. [148], which examined the tissue distribution of the oestrogen receptor in the ERKO mouse, comments on altered development of the male reproductive tract in these animals. The paper by Eddy et al. [149] describes male infertility and alteration of spermatogenesis in the ERKO mouse, but the authors specifically state that there were no remarkable differences in the anatomy or histology of the seminal vesicles or prostate as compared with the wild-type mouse. A male patient with oestrogen resistance caused by a mutation of the oestrogen receptor gene has been described [150]. This patient had normal male genitalia with bilateral descended testes and a normal-size prostate. Another male patient, with an aromatase deficiency [151], had a normal-size penis, but small testes, and a very low sperm count. For neither patient was sufficiently detailed information provided to adequately assess the full impact of the lack of oestrogen action on the structure and function their reproductive tracts. It would appear that oestrogens are not essential for the development of the gross anatomical structures of the tract [145], but they are necessary for physiological regulation of the structures. Clearly, then, males are potentially at risk for endocrine disruption not only by environmental androgen agonists/antagonists but also by environmental oestrogen agonists/antagonists.

The mechanisms of steroid hormone and xenobiotic action

Steroid hormone receptors

The steroid hormone receptors are members of a superfamily of ligand-inducible nuclear receptors that regulate hormone-responsive genes by controlling the rate of transcription initiation [121, 152, 153]. After becoming activated by binding ligand, the steroid receptors bind to specific response elements on DNA and then interact with components of the basal transcription machinery, either directly or through coactivators or other proteins [152–154]. These processes are thought to facilitate the formation of RNA polymerase II initiation complexes. Furthermore, information is becoming available on cross-talk between membrane and nuclear receptor systems [153, 155] and on the activation of genes by nonliganded receptors [156]. These factors add a greater degree of complexity to the regulation of genes by steroid hormones. The literature on the mechanisms of steroid hormone action has been reviewed extensively [121, 153, 156]; the reader is referred to these reviews for a more detailed analysis of the topic.

Of particular interest in this review is the binding of the steroid hormone or xenobiotic to the steroid receptor. An excellent and detailed treatment of this process and its consequences is presented by Katzenellenbogen et al. [153]. The sequences that constitute the ligand-binding pocket of the receptor are thought to be disorganized in the absence of the ligand. Recent X-ray and crystallographic data have confirmed this expectation [157, 158]. During receptor-ligand interaction, the receptor conforms to the shape of the ligand, and flexible ligands could have their conformation altered upon binding to the receptor [157, 158]. Since the ligand can control the shape of the receptor, it may also control its function by inhibiting or stimulating the coupling of the receptor-ligand complex to its effector, that is the sum of all the other components with which the complex interacts at each regulated gene [153]. In light of these considerations, one might anticipate that environmental xenobiotics that bind to steroid hormone receptors do not all result in conferring the same shape on their cognate receptor as does the physiological ligand. Those that do not are likely to result in disruption of normal receptor function. Since each environmental pollutant (or class of pollutants) probably results in a unique alteration in the conformation of the receptor to which it binds, unique alterations in function probably occur.

In this review, I have used the generic term 'oestrogen receptor'. However, an alternative oestrogen receptor (ER) has been cloned [159] and termed ERβ to distinguish it from the previously cloned oestrogen receptor, ERα. ERβ has been detected in several tissues, including the granulosa cells of the ovary, the prostate and epididymis using immunochemical and other means [54, 148, 160, 161]. ERβ and ERα can form heterodimers [162], both bind to DNA in a similar manner [163, 164], and similarities and differences in their ability to activate genes exist [165, 166]. Although ERα and ERβ have similar affinity for several compounds, ERβ has a

greater affinity for the phytooestrogen genistein than does ERα [148]. Whether ERβ has a higher affinity for other phytooestrogens or for oestrogenic environmental toxicants than does ERα remains to be determined. What differential roles, if any, are played by ERα and ERβ in the regulation of reproductive (and other) precesses remains to be explored.

Steroid-binding proteins

Data indicate [27] that xenobiotics not only interact selectively and specifically with steroid hormone receptors but also bind to the steroid-binding proteins ABP and SHBG. ABP is produced by the Sertoli cells of the testis and is primarily confined to the testis-epididymis compartment [167], whereas SHBG is produced by the liver and is present in the circulatory system [168]. Both of these proteins bind androgens with high affinity, and oestrogens with lower affinity [169]. ABP and SHBG are products of the same gene [168], but they are glycosylated differently [170]. These proteins were long considered to be steroid transport proteins whose function was to regulate the free levels of steroids or to sequester sex steroids into specific compartments [168]. However, over the years there have been several reports indicating that these proteins could be detected within cells, and several laboratories have now clearly established that receptors for these proteins are present on the plasma membranes of a variety of tissues (see refs 168 and 171 for reviews). The presence of such receptors adds a new dimension in considering how steroid hormones (and xenobiotics) act.

Little is known about the function of the membrane receptors for ABP/SHBG in physiological processes. The original speculation that the membrane recognition system for ABP/SHBG is involved in steroid transport may not be correct in view of recent studies [168, 171]. Functional data are accumulating that point to the ABP/SHBG receptor being a member of the G protein-linked class of receptors [168]. The first data were those of Rosner and co-workers, who showed a modest SHBG-5α-DHT-dependent increase in cyclic adenosine 3′,5′-monophosphate (cAMP) in the prostate cancer-derived LNCaP cells [168]. They have recently demonstrated a robust (700%) oestradiol-induced increase in intracellular cAMP in cells derived from benign hyperplastic prostate tissue [172]. They provide convincing evidence that this increase in cAMP is mediated through interaction of SHBG-steroid with its receptor. Other groups have shown ABP/SHBG-steroid stimulation of adenylyl cyclase in MCF-7 cells [168].

Data also demonstrate that the cAMP induced by the interaction of ABP/SHBG-steroid with the cell membrane receptor modulates growth of a prostate cancer cell line [173]. Growth was enhanced by inhibiting protein dephosphorylation with the protein phosphatase inhibitor okadaic acid, further implicating cAMP in the process. Using MCF-7 cells, Fortunati et al. [174] showed that growth was induced with oestradiol, but growth was inhibited by cAMP generated by the SHBG-oestradiol complex. Since pleiotropic effects of cAMP on growth are known [175], it is not surprising that opposite effects were seen in the above systems. SHBG has been shown to be involved in mediating prostate androgen receptor action [176], thus providing evidence for cross-talk between the ABP/SHBG receptor and the steroid receptor pathways.

The presence of the ABP/SHBG receptor system clearly presents another pathway that needs to be considered in examining the mechanisms by which xenobiotics might disrupt reproductive function. It has been demonstrated that several xenobiotics bind to SHBG and to ABP while others do not bind [27]. Likewise, there are xenobiotics that do and do not bind to the oestrogen and androgen receptors [27]. The xenobiotics that bind to SHBG or ABP could inhibit or stimulate a signal transduction cascade by participating in the ABP/SHBG receptor system. Binding of xenobiotics to ABP or SHBG could also decrease the availability of xenobiotics to the steroid nuclear receptors, thus lessening the potential endocrine-disrupting effects they might cause through that pathway. On the other hand, failure of xenobiotics to bind to ABP or SHBG would increase their availability to steroid nuclear receptors. In that case, the xenobiotics would have a greater apparent bioactivity than if they were bound to steroid transport proteins. This could be compared with DES, whose high oestrogenic potency and endocrine-disrupting effects are attributed, at least in part, to the fact that it does not bind to SHBG [177].

A survey of the literature uncovered only four papers in which binding of xenobiotics to ABP/SHBG was examined [27, 49, 178, 179]. One deals with binding of pyrethroids to SHBG [49] and another deals with binding of several xenobiotics to the proteins [27]. A third paper examined the ability of SHBG to attenuate oestrogen-stimulated *Lac Z* activity [178]. The fourth paper studied the ability of human serum (which contains SHBG) to influence the entry of several xenobiotics into MCF-7 cells and to bind to the oestrogen receptor [179]. The latter group also performed in vivo studies in mice [179]. They selected a xenobiotic to administer based on their in vitro studies; however, mice do not have circulating SHBG [180], so the results of these studies are difficult to evaluate.

Summary and conclusions

The above survey provides considerable data from epidemiological and experimental studies that clearly implicate environmental chemicals in the disruption of

reproductive function in animals. Direct evidence that environmental contaminants pose a risk to reproductive health of the human population is scant. However, this is exactly what one would anticipate. The large-scale production and dissemination of the compounds that we now know are reproductive toxicants in animals has only taken place during the last 50 or so years. This is far too short a horizon to expect to see dramatic reproductive consequences in humans whose life span, in contrast to that of the animals discussed above, exceeds that horizon. Only two or three human generations have reached reproductive age since the initial introduction of man-made chemicals into the environment. The first generation was probably exposed to too low a burden of the reproductive toxicants to have an effect.

Although some xenobiotics such as DDT and PCBs are no longer used in developed countries, their persistent nature [105–107] means that large quantities of these chemicals still contaminate the environment. The production of new chemicals that are being released into the environment may, potentially, be as hazardous to reproduction as the ones they are replacing. We are becoming aware of the fact that many compounds present in items used daily by humans, such as certain plastics and detergents, are endocrine disruptors in animals and in in vitro systems. These compounds were not previously recognized as reproductive toxicants. There are thousands of other compounds in the environment that have not been tested for their effects on reproduction; many of these may prove to be endocrine disruptors. The exposure of humans to environmental toxicants that may be hazardous to their reproductive systems is, therefore, likely increasing. Also of importance is the fact that many toxicants accumulate in fatty tissues of the body. Therefore, continued exposure of human populations to ever increasing numbers and amounts of environmental toxicants may have accumulative effects on reproductive health. These effects may not manifest themselves until future years.

As the DES case clearly demonstrates, delayed effects of reproductive toxicants can be expected. Human foetuses or prepubertal or pubertal humans may already have been exposed to critical doses of environmental endocrine-disrupting xenobiotics, the effects of which will not become obvious for many years. The report of girls entering puberty precociously [181], of declining sperm counts, of male genital anomalies, of increasing incidences of testicular cancer and of a decline in the ratio of male to female births, [4, 8, 104] may be early indicators of later more serious reproductive problems arising from environmental endocrine disruptors.

A major critic of the concern about the endocrine-disrupting effects of xenobiotics is Safe [182]. He contends that the effects of environmental xenobiotics to which we are exposed would be swamped by endogenous and dietary oestrogens. This point is worthy of consideration, but it fails to take into account the possibility that some environmental endocrine-disrupting agents are antiandrogens/androgens. It also does not recognize the possibility that xenobiotics may act not only through steroid receptors but also through steroid-binding protein pathways. Furthermore, the interaction of xenobiotics with steroid receptors or binding proteins may not result in the same kind of transactivation as occurs with physiological ligands. If dissociation of a xenobiotic from a receptor or binding protein is slower than that of endogenous oestrogens or androgens [27] or if nuclear retention of the receptor-xenobiotic complex is longer [50], persistent effects could occur that would make up for the lower affinity of xenobiotics for these proteins. In addition, in vivo metabolism of xenobiotics can activate them to forms that may have a much higher affinity for the receptors and binding proteins than we are currently aware of.

It must be remembered that we are not exposed to each environmental toxicant individually, but rather we are exposed, simultaneously, to the sum of all xenobiotics present in the environment. These environmental toxicants can accumulate in foodstuffs and in ourselves, thereby augmenting our exposure to them. There are numerous known and unrecognized agonists and antagonists of reproductive hormones in the environment which, working together, have the potential to disrupt the physiological regulation of the development and function of the reproductive systems of animals and humans. Since xenobiotics can act through several steroid-dependent and other (Ah receptor, metabolizing enzymes, thyroid hormone receptor etc.) pathways, the possibility exists for augmenting the effects of even low concentrations of weak environmental hormone agonists and antagonists.

It is concluded from the data and from the considerations presented that the hypothesis that man-made chemicals in the environment pose a risk to human reproductive health is highly plausible.

Future directions

What must be done in the very near future is to identify environmental toxicants that disrupt the normal development and function of the reproductive system. Only 40 or so environmental pollutants have been identified as endocrine disruptors [1, 4]. Most of these have been identified not as the result of logical or exhaustive screening processes but rather by serendipity. Several in vitro systems have been developed recently that should allow for the rapid screening of large numbers of potential disruptors of reproduction [27, 80, 178, 179]. Other assays [172] can be modified to determine if xenobiotics

act through ABP/SHBG-dependent pathways. The in vitro assays should be considered a first step in evaluating xenobiotics for their endocrine-disrupting potential. And it should be realized that such assays can produce false negatives and false positives since in vivo metabolic products of the compounds may be the actual agonist/antagonist.

The in vitro studies will not tell us what the compounds actually do to reproductive systems. To determine this, in vivo studies must be conducted in animals. The selection of the compounds to be tested in vivo would be guided by the results of the in vitro studies. It makes sense from the standpoint of feasibility, time and money to initially treat animals with large doses (e.g. mg/kg/day) of the test compounds over a short period of time (e.g. 2 weeks) to determine if effects can be demonstrated. It might be argued that if one gives an animal a large enough dose of a toxic compound, one is bound to see something. This argument, however, is fallacious, because one is not looking for general effects, but for specific effects on the reproductive system. Giving a large dose of a single environmental toxicant to an experimental animal for a short period of time may mimic a lifetime of exposure of the animal (or human) to the numerous potentially hazardous toxicants in the environment. Once an effect, if any, has been demonstrated, further experiments to evaluate dose-response criteria can be designed more logically. An appropriate guideline for determining the dose of environmental toxicant to administer to an experimental animal would be to base it on the relative affinity of the environmental toxicant for the steroid receptor or binding protein with which it interacts and on the metabolic clearance rate of the xenobiotic from the body. The latter is seldom known. Thus, the relative affinity parameter appears to be the best available guideline for deciding the dose to administer. Since the relative affinity of environmental toxicants for the receptors and binding proteins is orders of magnitude lower than that of the physiological ligands [27, 82], it is obvious that the milligram-per-day doses that we suggest are necessary to approximate effective concentrations of physiological steroids, which are present in concentrations of picograms to nanograms per millilitre of serum or per gram of tissue [183, 184]. Consequences of the treatments to be examined should include gross anatomical and histological alterations of the reproductive systems of the offspring of treated pregnant females, and of animals treated at various stages of development so that critical periods during which various reproductive parameters are affected can be determined. The effects of treatment on such parameters as spermatogenesis and fertility should also be evaluated.

It is also necessary to determine the specific genes that are activated/inhibited by the xenobiotics following in vivo administration using such techniques as differential display polymerase chain reaction [185]. It is the products of these regulated genes that result in the structural and functional disruption of the male and female reproductive systems. Thus, a multifaceted approach using in vitro and animal [84, 186] models is needed to evaluate the end-points of environmental toxicant action and the mechanisms by which these are brought about.

Physicians and other health care professionals need to have a heightened awareness of the fact that clinical signs, such as precocious puberty, decreased sperm counts, anatomical anomalies of the reproductive tract, decreased fertility and so on, may be attributable to the endocrine-disrupting effects of environmental pollutants. State and national centres should be established to which these data can be reported. In-depth surveys need to be conducted on people living in, or who have lived in, highly polluted environments to determine if long-term effects on reproductive function are occurring. Since clear-cut effects of environmental toxicants on the reproductive systems of animals living in such areas have been demonstrated, it would be unexpected if such effects did not also occur in humans. In the final analysis, it would be rash to dismiss the possibility that environmental toxicants have harmful effects on human reproductive health unless and until evidence proves the contrary.

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