

***The Endocrine Disrupting Activities
of Major Industrial Chemicals -
the Phthalate Esters and
4-Nonylphenol***

A thesis submitted for the degree of Doctor of Philosophy

by

Catherine Anne Harris

The Fish Physiology Research Group, Department of Biological Sciences,
Brunel University

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Declaration

The work described in this thesis was carried out between 1996 and 2000 at Brunel University (Uxbridge, Middlesex), and the NERC Centre for Ecology and Hydrology (Windermere, Cumbria). This work was carried out independently and has not been submitted for any other degree.

Abstract

*Brunel University, Uxbridge, Middlesex, UB8 3PH
Department of Biological Sciences
Catherine Anne Harris*

A number of widely used industrial chemicals have been shown to possess endocrine-disrupting properties. In this thesis, a series of in vitro tests, and an in vivo reproductive performance test with fathead minnows, were used to clarify the extent of estrogenic activity exhibited by the phthalate esters - a class of compound hitherto referred to as 'estrogenic'.

Using a recombinant yeast estrogen screen, I demonstrated that a small number of commercially available phthalates showed extremely weak estrogenic activity. The most potently estrogenic phthalate of those tested was BBP, which was approximately one million-fold less potent than 17 β -estradiol. The phthalates which were estrogenic in the yeast screen were also mitogenic on estrogen-responsive human breast cancer cells (MCF-7 and ZR-75 cell lines). The most prolifically used phthalate, DEHP, was not estrogenic in any of these assays. The small number of metabolites of phthalate metabolites tested (including MBuP, MBzP, MEHP and MnOP) were also not estrogenic in the recombinant yeast assay.

The ability of BBP (as the most potently estrogenic phthalate in vitro) to induce a vitellogenic response (an indicator of estrogen exposure) in fathead minnows (*Pimephales promelas*) exposed via the water was assessed. No induction of vitellogenin was observed, indicating that 100 μ g BBP/L (a concentration higher than would normally be found in the environment) is not estrogenic to this species of fish under the conditions employed for this experiment. In the same study, fecundity of breeding pairs of fathead minnows was assessed; exposure to BBP was not found to affect reproductive performance in these fish.

A possible alternative mechanism of action of the way in which the phthalates induce frequently reported reproductive disorders was observed. Some of the phthalates, and, notably, some of their metabolites, were demonstrated to act as anti-androgens in a recombinant yeast androgen assay.

4-Nonylphenol is another industrial chemical which is used in large volumes, and due to the nature of its use (mainly in detergent formulations), is discharged into water systems via sewage effluents. This chemical has been shown to be estrogenic to fish at the concentrations at which it has been detected in the environment.

4-Nonylphenol was tested for its ability to affect plasma and pituitary gonadotropin levels in female recrudescing rainbow trout (*Oncorhynchus mykiss*). Plasma and pituitary levels of FSH were suppressed in fish exposed to 10 and 100 μ g 4-NP/L. In addition, FSH gene expression was reduced in these fish, and also in the fish exposed to 1 μ g 4-NP/L. Pituitary LH content and gene expression of this hormone were suppressed in the fish exposed to 100-, and 10- and 100 μ g 4-NP/L respectively. Gonadal development in vertebrates is regulated by FSH. Ovarian development ceased in the fish exposed to 100 μ g 4-NP/L, possibly as a result of the suppression of FSH synthesis and/or release in these fish.

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CONTENTS

Title Page.....	i
Declaration.....	ii
Thesis Abstract.....	iii
Acknowledgements.....	iv
Contents.....	v
List of tables and figures.....	ix
1 INTRODUCTION.....	1
1.1 ENVIRONMENTAL ENDOCRINE DISRUPTION.....	2
1.1.1 Evidence for reproductive abnormalities in humans.....	2
1.1.2 Evidence for reproductive abnormalities in wildlife.....	3
1.1.2.1 Mammals.....	4
1.1.2.2 Birds.....	4
1.1.2.3 Reptiles.....	5
1.1.2.4 Fish.....	6
1.1.2.5 Invertebrates.....	9
1.2 GONADAL SEX STEROIDS.....	10
1.2.1 Class, structure, and activity.....	10
1.2.1.1 Estrogens.....	13
1.2.1.2 Androgens.....	13
1.2.1.3 Progestogens.....	15
1.2.2 Activation of the receptor.....	15
1.2.3 The role of sex steroid hormones in fish reproduction.....	17
1.2.4 Occurrence of steroidal compounds in the aquatic environment.....	18
1.3 NATURALLY OCCURRING XENOESTROGENS IN THE ENVIRONMENT.....	20
1.4 SYNTHETIC HORMONE MIMICS IN THE ENVIRONMENT.....	23
1.4.1 Organohalogen compounds.....	24
1.4.1.1 Polychlorinated biphenyls.....	24
1.4.1.2 Dioxins and furans.....	27
1.4.2 Pesticides.....	28
1.4.3 Surfactants.....	32
1.4.4 Plasticisers and other plastics components.....	32
1.5 AIMS	34

2	GENERAL MATERIALS AND METHODS	35
2.1	METHODS EMPLOYED IN THE ASSESSMENT OF THE ENDOCRINE DISRUPTING POTENTIAL OF CHEMICALS.....	36
2.1.1	Recombinant yeast assays.....	37
2.1.1.1	Preparation and storage of medium components.....	38
2.1.1.2	Assay procedure.....	40
2.1.1.3	Specificity and sensitivity.....	41
2.1.1.4	Calculation of potencies of test chemicals.....	44
2.1.2	Mammalian breast cancer cell lines.....	44
2.1.2.1	Assay procedure.....	44
2.2	THE RADIOIMMUNOASSAY.....	46
2.2.1	The standard curve.....	46
2.2.2	Separation methods.....	47
2.2.3	Counting.....	49
2.3	PROTEIN RADIOIMMUNOASSAYS.....	50
2.3.1	Protein assay buffer (PAB).....	50
2.3.2	Purification of label.....	50
2.3.3	The vitellogenin radioimmunoassay.....	50
2.3.3.1	Vitellogenin standard.....	50
2.3.3.2	Vitellogenin antibody.....	52
2.3.3.3	Iodination of vitellogenin.....	52
2.3.3.4	Routine assay plan.....	52
2.3.3.5	Protocol.....	53
2.3.4	The FSH radioimmunoassay.....	54
2.3.4.1	FSH standard.....	54
2.3.4.2	FSH antibody.....	54
2.3.4.3	Iodination of FSH.....	54
2.3.4.4	Routine assay plan.....	54
2.3.4.5	Protocol.....	54
2.3.5	The LH radioimmunoassay.....	55
2.3.5.1	LH standard.....	55
2.3.5.2	LH antibody.....	55
2.3.5.3	Iodination of LH.....	55
2.3.5.4	Routine assay plan.....	55
2.3.5.5	Protocol.....	56
2.4	STEROID RADIOIMMUNOASSAYS.....	56
2.4.1	Steroid assay buffer (SAB).....	56

2.4.2	Routine assay plan.....	56
2.4.3	Assay protocol.....	57
2.5	EXTRACTION AND ANALYSIS OF WATER SAMPLES.....	59
2.5.1	Collection and storage.....	59
2.5.2	Extraction.....	59
2.5.3	Analysis of extracted samples.....	60
3	AN ASSESSMENT OF THE ENDOCRINE DISRUPTING POTENTIAL OF PHTHALATE ESTERS.....	62
3.1	INTRODUCTION.....	63
3.1.1	The abundance of phthalates in the modern environment.....	63
3.1.1.1	Fate of phthalates in the aquatic environment.....	66
3.1.1.2	Fate of phthalates in the terrestrial environment.....	67
3.1.1.3	Degradation of phthalates in the environment.....	67
3.1.1.4	Exposure of humans to phthalates.....	68
3.1.1.5	Exposure of aquatic organisms to phthalates.....	69
3.1.1.6	Metabolism and bioaccumulation.....	70
3.1.1.7	Metabolism and bioaccumulation of phthalates in aquatic organisms.....	70
3.1.2	The reported endocrine activity of phthalates.....	72
3.1.2.1	Estrogenic activity in vitro.....	73
3.1.2.2	Estrogenic activity of phthalates in vivo.....	74
3.1.2.3	Anti-androgenic activity of phthalates.....	75
3.2	METHODS.....	76
3.2.1	Recombinant yeast assays.....	76
3.2.2	In vitro assays using mammalian breast cancer cell lines.....	78
3.2.3	Assessment of the estrogenic activity of BBP in vivo.....	78
3.2.3.1	Experimental design.....	78
3.2.3.2	Chemical analysis.....	80
3.2.3.3	Assessment of estrogenicity of the tank water.....	81
3.2.3.4	Statistical analyses.....	81
3.3	RESULTS.....	81
3.3.1	Estrogenic activity of phthalates in a recombinant yeast assay.....	81
3.3.2	Estrogenic activity of phthalates assessed using mammalian breast cancer cell lines.....	90
3.3.3	Anti-androgenic activity of phthalates in a recombinant yeast assay	90

3.3.4	Assessment of the estrogenic activity of BBP in vivo	
	in fathead minnows.....	94
3.3.4.1	Chemistry and estrogenic activity of the tank	
	water samples	94
3.3.4.2	Plasma vitellogenin concentrations.....	96
3.3.4.3	Fecundity.....	98
3.4	DISCUSSION AND CONCLUSIONS.....	98
4	<i>THE IMPACT OF 4-NONYLPHENOL ON THE</i>	
	<i>NEUROENDOCRINE REGULATION OF</i>	
	<i>REPRODUCTION IN MATURING</i>	
	<i>FEMALE RAINBOW TROUT</i>	110
4.1	INTRODUCTION.....	111
4.1.1	The chosen model xenoestrogen: 4-Nonylphenol.....	111
	4.1.1.1 Environmental occurrence.....	111
	4.1.1.2 Estrogenic activity.....	118
4.1.2	The brain-pituitary-gonadal (BPG) axis.....	120
	4.1.2.1 The influence of steroids on the regulation of	
	gonadotropins in fish.	122
4.2	METHODS.....	125
4.2.1	Experimental design.....	125
	4.2.1.1 Concentrations of 4-NP selected.....	125
	4.2.1.2 Experimental protocol.....	126
	4.2.1.3 Selection of fish.....	128
	4.2.1.4 Dosing of experimental tanks.....	128
	4.2.1.5 Sub-lethal sampling.....	129
	4.2.1.6 Terminal sampling.....	129
4.2.2	Extraction of pituitary glands.....	129
	4.2.2.1 Dot blot hybridisation.....	130
4.2.3	Statistical analyses.....	132
4.3	RESULTS.....	132
4.3.1	Actual concentrations of 4-NP to which the fish were exposed.....	133
4.3.2	The effect of 4-NP on general growth and reproductive endpoints of	
	maturing female rainbow trout.....	137
	4.3.2.1 Mortality.....	137
	4.3.2.2 Weight and length.....	137
	4.3.2.3 GSI and HSI.....	141

4.3.2.4	Vitellogenin.....	141
4.3.2.5	17 β -Estradiol.....	145
4.3.2.6	Testosterone.....	145
4.3.3	The effect of 4-NP on gonadotropin concentrations in maturing female rainbow trout.....	148
4.3.3.1	Plasma FSH concentrations.....	148
4.3.3.2	FSH content and gene expression in the pituitary.....	148
4.3.3.3	Plasma LH concentrations.....	152
4.3.3.4	LH content and gene expression in the pituitary.....	152
4.3.4	A comparison between the fish maintained in the solvent control versus those in the absolute control tanks.....	156
4.6	DISCUSSION AND CONCLUSIONS.....	158
5	GENERAL DISCUSSION.....	168
	LIST OF MY PUBLICATIONS TO DATE.....	174
	REFERENCES.....	175

List of Figures and Tables

<i>Figure 1.1</i>	<i>The core structures of the steroid hormones.....</i>	<i>11</i>
<i>Figure 1.2</i>	<i>The pathway of steroid biosynthesis in teleosts.....</i>	<i>12</i>
<i>Figure 1.3</i>	<i>Schematic of a teleost ovarian follicle.....</i>	<i>14</i>
<i>Figure 1.4</i>	<i>Schematic of gene activation in the cell nucleus.....</i>	<i>16</i>
<i>Figure 1.5</i>	<i>Structure of PCBs, dioxins and furans.....</i>	<i>25</i>
<i>Figure 2.1</i>	<i>Schematic of the estrogen inducible expression system in yeast.....</i>	<i>39</i>
<i>Figure 2.2</i>	<i>Specificity of the recombinant yeast estrogen assay.....</i>	<i>42</i>
<i>Figure 2.3</i>	<i>Specificity of the recombinant yeast androgen assay.....</i>	<i>43</i>
<i>Figure 2.4</i>	<i>Calculation of potency of a chemical in the recombinant yeast assay.....</i>	<i>45</i>

Figure 2.5	A typical standard curve obtained from a radioimmunoassay.....	48
Figure 2.6	Purification of a radiolabelled protein/hormone.....	51
Figure 2.7	Illustration of the extraction of water samples	61
Table 3.1	Details of the high volume use phthalates studied.....	63
Figure 3.1	Structures of the high volume use phthalates.....	65
Figure 3.2	Structures of primary metabolites of some of the phthalates.....	71
Table 3.2	Phthalates tested in the yeast estrogen assay which are not used in high volumes in Europe.....	77
Figure 3.3	Adult male and female fathead minnows.....	79
Figure 3.4	Estrogenic activity of high volume use phthalates in a recombinant yeast assay.....	82
Figure 3.5	Estrogenic activity of commercially available (low volume) phthalates in a recombinant yeast assay	84
Figure 3.6	Phthalates with no estrogenic activity in a recombinant yeast assay.....	85
Figure 3.7	The activity of DTDP and o,p'-bisphenol-A (a contaminant in the DTDP mixture) in a yeast assay.....	87
Figure 3.8	Development of the BBP response curve over time.....	88
Table 3.3	Potencies of the phthalates in the yeast assay	89
Figure 3.9	Proliferation of ZR-75 cells incubated with phthalates.....	91
Figure 3.10	Proliferation of MCF-7 cells incubated with phthalates.....	92
Figure 3.11	Anti-androgenic activities of phthalates and their metabolites in a recombinant yeast assay.....	93
Table 3.4	Actual concentrations of BBP and 4-NP measured in the exposure tanks.....	94
Figure 3.12	Estrogenic activities of tank water samples in which fathead minnows were maintained.....	95
Figure 3.13	Plasma vitellogenin concentrations in fathead minnows exposed to BBP and 4-NP.....	97
Figure 3.14	Fecundity of fathead minnows exposed to BBP and 4-NP.....	99
Figure 3.15	In vivo anti-androgenic effects of DBP.....	104
Figure 4.1	Structure of a typical alkyphenol.....	112
Figure 4.2	Structure of a typical alkyphenol ethoxylate.....	113
Figure 4.3	Pathway of degradation of APEOs during sewage treatment.....	114
Figure 4.4	Simplified diagram of the BPG axis.....	121
Figure 4.5	Annual profiles of FSH and LH relative to GSI in female rainbow trout.....	123

Figure 4.6	The tank set-up at Windermere.....	127
Figure 4.7	Alignment of the nucleotide sequence of isolated rainbow trout FSH with that of chum salmon FSH cDNA.....	131
Table 4.1	Actual concentrations of 4-NP detected in the tanks over the period of the study.....	133
Figure 4.8	Actual concentrations of 4-NP in the experimental tanks over the period of the study.....	134
Figure 4.9	The estrogenic activity of the tank water samples in the yeast screen.....	136
Figure 4.10	Weight of the fish over the duration of the experiment.....	138
Figure 4.11	Length of the fish over the duration of the experiment.....	139
Figure 4.12	Coefficient of condition of the fish throughout the experiment...	140
Figure 4.13	Gonadosomatic index of the fish at the end of the experiment....	142
Figure 4.14	Hepatosomatic index of the fish at the end of the experiment.....	142
Figure 4.15	Typical ovaries collected from the fish prior to the start, and at the end of the experiment.....	143
Figure 4.16	Plasma vitellogenin concentrations throughout the experiment.....	144
Figure 4.17	Plasma E2 concentrations throughout the experiment.....	146
Figure 4.18	Plasma testosterone concentrations after 6 weeks exposure....	147
Figure 4.19	Plasma FSH concentrations throughout the experiment.....	149
Figure 4.20	Pituitary FSH content at the end of the experiment.....	150
Figure 4.21	FSH gene expression in the pituitaries at the terminal sample.....	151
Figure 4.22	Plasma LH concentrations throughout the experiment.....	153
Figure 4.23	Pituitary LH content at the end of the experiment.....	154
Figure 4.24	LH gene expression in the pituitaries at the terminal sample.....	155
Figure 4.25	Comparison between fish maintained in the absolute control and solvent control tanks.....	157
Figure 4.26	Schematic of GTH profiles in relation to oocyte development.....	161

CHAPTER 1

INTRODUCTION

1.1 ENVIRONMENTAL ENDOCRINE DISRUPTION

The issue of endocrine disrupters in environmental research is relatively young. One of the principal reasons for this is the subtlety of potential effects of such chemicals at the concentrations at which they are normally encountered in the environment, and therefore the length of time between the release of industrial chemicals and the realisation that their presence in the environment may be causing adverse effects in humans and/or wildlife. Indeed, it is possible that research efforts would have been even slower getting off the ground were it not for extreme exposure situations, culminating in distinct debilitating effects on humans or wildlife, such as when DES (diethylstilbestrol) was given to expectant mothers in the 1940's through to the 1970's (Stillman, 1982), and point source discharges or spills of highly toxic effluents into lakes and coastal areas (Guillette et al, 1996; Anderson et al, 1975).

As a result of the youthfulness of this topic, there are presently few definitive statements which we can make about the effects of environmental endocrine disrupters on environmentally-exposed individuals. Limited data are available regarding the effects of some specific chemicals at the individual level in the laboratory. However, the big gap in our knowledge is in our understanding of the effects of endocrine disrupting chemicals, either singly or as mixtures, on wildlife (that is, wild animals, rather than laboratory animals) at the individual, let alone the population, level. There is a great deal of work to be undertaken before we can draw any conclusions as to the real threat of these chemicals to our ecosystems.

1.1.1 Evidence for reproductive abnormalities in humans

In recent years there appears to have been a striking increase in the incidences of reproductive disorders in humans, particularly in males. Abnormalities such as cryptorchidism, hypospadias, and testicular and prostate cancer are among the adverse reproductive health conditions occurring with increased frequency (as discussed by Toppari et al, 1996). These cannot unequivocally be linked to the presence of endocrine disrupting chemicals (EDCs) in the environment; although their increasing incidence is correlated somewhat with a rise in exposure of the human population to industrial chemicals, the relationship hypothesised between

the two is primarily by association. The majority of these disorders are thought to be related to malfunctions occurring during foetal development, and are similar to effects observed in the male offspring of DES-treated mothers (Gill et al, 1979; Gill, 1989). It is also known that lipophilic organic chemicals can accumulate in body fat, and can be released during pregnancy (for example into placental fluid) as energy resources are exploited, thus increasing potential foetal exposure to EDCs. Thus, a plausible link between man-made organic chemicals and the apparent decrease in male reproductive health can be made, but evidence proving causality is not available presently.

A possible reduction in sperm count, quality, and quantity, since the 1940's has also been discussed (Carlsen et al, 1992; Auger et al, 1995). While this too is not definitively a result of increased exposure to environmental estrogens - indeed these are controversial findings and they have been disputed on the basis of statistical inaccuracies (Olsen et al, 1995) - Sharpe and Skakebaak (1993) hypothesised that the phenomenon may be linked to in utero exposure of humans to xenoestrogens.

It is not only males which have appeared to show an increase in reproductive anomalies; there has also been a steady rise in the incidence of breast cancer in the past three or four decades (Wolff et al, 1993). This too is a disease which in some cases can be enhanced by estrogens, and hence estrogen-mimicking chemicals could play a role in the aetiology of this disease.

1.1.2 Evidence for reproductive abnormalities in wildlife

There are a limited number of studies describing conclusive incidences of endocrine disruption in wild populations of any species. Certainly, there are few which can, beyond doubt, attribute effects at the level of the community to such contaminants. This is predominantly a result of the complex variety of environmental influences on wildlife ecosystems. Some of the studies attributing adverse reproductive effects in certain organisms to endocrine disrupting environmental pollutants - mainly as a result of accumulation of circumstantial evidence, but in some cases strengthened by similar effects observed in laboratory situations - are described below.

1.1.2.1 Mammals

One of the earlier reports concerning population-level effects of what are now known to be, amongst other things, endocrine active pollutants, the polychlorinated biphenyls (PCBs), involved the collapsing number of common seals in one area of the Wadden Sea (the Netherlands) (Reijnders, 1986). It was known that PCB levels in seals inhabiting this area were elevated, and it was hypothesised that this may be affecting their reproductive capacity. Captive seals fed on fish from this area showed a higher rate of reproductive failure compared to control seals fed on uncontaminated fish, thus demonstrating an association between contaminants normally encountered by seals via their food source, and a physiological response in these mammals (Reijnders, 1986).

More recently, the demise in number of the Florida Panther has been associated with reproductive impairment of this species as a result of exposure to toxicants such as p,p'-DDE and PCBs (Facemire et al, 1995). Male panthers were found to contain E2 in their serum at concentrations similar to that observed in females, in addition to having low sperm counts and poor sperm quality, and incidences of cryptorchidism.

Polar bears in Svalbard have also been reported to show signs of some kind of (androgenic) endocrine disruption, whereby a 1.5% incidence of "pseudohemaphroditism" - females exhibiting either a 20 mm penis (in addition to normal female genitalia), or clitoral hypertrophy - was observed (Wiig et al, 1998). This scenario is perhaps to be expected in Arctic and Antarctic regions if endocrine disruption is indeed a reality, since organic chemicals are known to migrate to and accumulate in colder environments (due to their lack of volatility at decreased temperatures), in addition to the fact that polar bears reside at the top of the food chain in these areas, consuming blubber-rich seals which themselves accumulate lipophilic pollutants. However, there were no indications of a population-level effect on the polar bears, and as yet no evidence of a lack of reproductive capacity of the pseudohermaphrodite females.

1.1.2.2 Birds

Some bird populations have shown evidence of declining numbers in relation to exposure to environmental contaminants. These have most frequently been fish-

eating species, feeding on fish inhabiting polluted waters, which are likely to accumulate toxic organic chemicals. However, some reports of birds affected by urban and agricultural pesticides, which may be acting via the endocrine system, have also been published (see Fry, 1995, for a review). One example is that of the Brown Pelican population on the Southern California coast. Up until 1970, a DDT manufacturing plant in the area discharged its waste into the ocean - leading to contaminated fish stocks (eg anchovies) on which the pelicans fed - and this was associated with falling pelican numbers. Around 1970, the plant changed its disposal practice, and deposited its waste into sanitary landfills. The subsequent improvement in eggshell condition and egg survival, culminating in an increased number of Brown Pelicans, was reported by Anderson et al (1975).

Another fish-eating bird species, also present in Southern California, is the California gull (*Larus californicus*), the embryos of which were reported to be feminised by DDT exposure (Fry & Toone, 1981). A controlled experiment was conducted by these scientists whereby gull eggs were injected with DDT at levels similar to those found in eggs of affected populations. This was found to induce adverse reproductive developmental effects on ovarian tissue, together with the production of oviducts in males. A link was suggested between these laboratory data and the observation of a skewed sex ratio (3.85:1 female:male) in the wild gull population (the eggs of which had been found to contain high concentrations of DDT), which in turn had led to female-female pairing at the nests of these birds.

Reproductive failure in aquatic birds inhabiting the Great Lakes region has also been correlated to exposure to organic, halogenated compounds such as PCBs, PCDDs and PCDFs, which caused, in particular, eggshell thinning (Giesy et al, 1994).

1.1.2.3 Reptiles

The most extensively studied reptilian population thought to be exposed to endocrine disrupting chemicals is the alligators in Lake Apopka. Studies were initiated to assess the population status following a spill of DDT and dicofol into the lake, which is also contaminated by sewage and surrounded by an area of agricultural activity which may result in toxic runoff into the lake. A lack of recruitment of alligators in this lake was noted, and Guillette and colleagues have

followed up this finding with several studies in an attempt to pin down the reasons behind this reproductive failure. In 1994, they reported the existence of male alligators with decreased testosterone concentrations, abnormally developed testes, and small phalli. In addition, female alligators were found to have increased E2 levels and abnormal ovaries. It was suggested that the effects were due to exposure to toxic chemicals in ovo, and were irreversible (Guillette et al, 1994). In unrelated studies, Rie et al (1999) have recently documented evidence of the negative impact of fuel and chemical spills on the reproductive system of the turtle. Although no evidence of excessive concentrations of organic pollutants in turtles was observed at the sites investigated, a mixture of contaminants was found, among them were some phthalates (thought to be weakly estrogenic, and recently also described as anti-androgenic) (Rie et al, 1999).

1.1.2.4 Fish

Clearly, if toxic organic chemicals are being discharged into aquatic systems, fish are a particularly vulnerable group of organisms. They have the potential to take up such toxins via several different pathways, and may be subject to bioconcentration of endocrine disrupters by virtue of the fact that they may be permanently surrounded by contaminated water. This water will pass over their gills, which are extremely efficient at removing chemicals from the water (particularly oxygen, of course). These chemicals enter the bloodstream, and reach most organs before passing through the liver (where they may be metabolized to less active chemicals). Fish may also receive exposure to toxicants through eating contaminated food, and since many fish species lie towards/at the top of the food chain, these chemicals may be found in relatively high concentrations in these organisms as a consequence of biomagnification.

Much of the work undertaken regarding the estrogenic nature of sewage effluents discharged into rivers in England was initiated by a chance discovery. Scientists investigating vitellogenin levels (a yolk protein precursor, normally only found in mature female fish) in rainbow trout kept at a MAFF fish farm were surprised to find detectable concentrations of this substance in male fish. The discovery was thought to be a result of contamination by an estrogenic component in the treated sewage effluent which was present in the water in which the fish were kept (Sumpter, JP, pers. comm.). This discovery preceded a survey by these

scientists of effluents at various sewage treatment works (STWs) around the UK. Data from these studies, in which fish were kept for 3 weeks in treated effluent, indicated that each of the 15 effluents in which the fish survived (in 15 others all the fish died) induced the estrogenic response of vitellogenin production in the male rainbow trout (Purdom et al, 1994). There was also a small amount of data in this paper describing the induction of this response downstream of the effluent discharge. The authors speculated that the chemical/s responsible may be ethynylestradiol, which is used in the contraceptive pill, and/or the alkylphenol ethoxylates used at that time in large quantities as surfactants in many products (eg detergents, pesticides).

Subsequently, Harries et al (1997) extended the studies of Purdom et al (1994), and investigated the persistence of estrogenic activity downstream of effluent discharges from six STWs in England. In three of the rivers, no estrogenic effect was observed but at the other three, some element of estrogenicity was maintained (albeit at reduced potency) at up to 1.5 km, 4.5 km, and more than 5 km downstream of the effluent discharges. Harries et al also studied the potency of various dilutions of effluent produced by Chelmsford and Harpenden STWs, and found that at dilutions of 25% and 50%, respectively, elevated plasma vitellogenin concentrations in male fish could be observed (Harries et al, 1999). To put these data into context, many British rivers contain high concentrations of effluent; for example, the River Lea has been shown to comprise more than 80% effluent during the drier summer months (Harries et al, 1999), and this figure is not atypical of many rivers in England.

With respect to reproductive and potential populational effects in wild populations of fish in these rivers, Jobling et al recently reported high incidences of intersex roach inhabiting stretches of river downstream of STWs in England. This study was able to correlate the increasing frequency and degree of intersex with the concentration of sewage effluent at the various sites (as measured by population equivalents of the respective STWs and average annual dilution of the effluent) (Jobling et al, 1998). Although the authors could not definitively state the sex of the intersex fish, it was suggested that they were feminised males as opposed to masculinised females, due to the number of "normal" males in a population being inversely proportional to the number of intersex fish. At some sites, 100% of macroscopic males were intersex to some degree. Although the impact of this finding on the successful reproduction and population endpoints is unknown

(studies are currently being undertaken to assess the reproductive capabilities of intersex fish), in general the phenomenon of intersex roach is thought to be rare. The first reported hermaphrodite roach was in 1909 (see Arme, 1965, for details), followed by the finding of one more individual specimen being reported by Arme (1965), and a microscopically intersex roach (1 out of more than 1000 specimens which had passed through the laboratory) was described by Jafri & Ensor (1979). The former two examples were macroscopic hermaphrodites (i.e. intersexuality could be observed by eye, without the need for any microscopic investigation of the gonad) and the background information concerning microscopically intersex roach (whereby the gonad is not obviously intersex, but microscopic investigation shows that it is, by virtue of the fact that oocytes are found in the testes) is less prevalent. In 1981, Sweeting reported an unusually high incidence of hermaphrodite roach in the settlement lagoons of 2 STWs discharging into the river Lea, and, interestingly, found the highest proportion (up to 20%) in older fish, therefore leading to the hypothesis that a cumulative effect might be occurring (Sweeting, 1981). A follow-up survey by Ensor & Tinsley described the persistence of this condition in 6.4% of one of the populations of roach examined, compared to no intersex at all in the control fish (Ensor and Tinsley, 1984).

In the US and Canada, although estrogenic sewage treatment effluents have been reported (Folmar et al, 1996), they appear to be less of a issue in these countries, perhaps as a result of the higher level of treatment at STWs in the US, but also because, by virtue of the size of many American rivers, effluents are in any case diluted to a greater degree than in the UK. Research into the endocrine disruption of fish has focused more on discharges eschewing from pulp and paper mills. The endocrine disrupting nature of this type of effluent was first reported by Howell et al (1980), who reported the masculinization of female mosquitofish downstream of a paper mill discharge, as compared to the normal development of this species upstream of the outflow. This masculinization took the form of both physical secondary sex characteristics (females possessed an elongated anal fin, or gonopodium, which is used by males in the deposition of sperm), together with male reproductive behaviour. In addition, males exhibited signs of precocious development (Howell et al, 1980). These effects were attributed to an androgenic component (of unknown nature) present in the effluent. Paper mill effluents such as these are known to contain phytoestrogens, for example β -sitosterol, and it has been suggested that these compounds may have been converted to androgenic compounds, thus rendering the effluent with

overriding masculinizing properties (Davis & Bortone, 1992). The initial study by Howell et al (1980) drew no conclusions as to the reproductive capabilities of the masculinized females, and it was, therefore, not possible to hypothesise as to potential population effects. More recently, Gagnon et al (1995) concluded that fish (in this case, white sucker) inhabiting stretches of river downstream of bleached kraft mill effluents invested less energy in reproduction, possessed smaller gonads, and exhibited an increased variability in fecundity, compared to uncontaminated rivers.

1.1.2.5 Invertebrates

A classic example of an unfolding story of endocrine disruption is the case of tributyl tin (TBT) inducing male characteristics in females of invertebrate populations. Smith first linked the occurrence of abnormal snails with pollution from marinas (Smith, 1981a), which was followed by him reporting a relationship between these abnormalities and contamination from antifouling paint on ships hulls (Smith, 1981b), after which this association was established by laboratory tests where imposex snails were produced following exposure to TBT (Smith, 1981c). In this latter report, there was apparently no impact on reproductive potential or population dynamics, and imposex was simply suggested to be a biological indicator of TBT contamination. However, certain invertebrate species (such as dog whelks) actually exhibited a reduction in numbers as a consequence of an imposex condition and the resultant inability to reproduce, and this was hypothesised as being a result of such organotin contamination (Spooner et al, 1991). The mechanism behind this observed imposex effect is currently unclear, although it is thought to be a result of increasing androgen levels, due to the inhibition of aromatase activity by TBT, as opposed to a direct TBT effect (Spooner et al, 1991; Bettin et al, 1996). More recently, Ronis & Mason (1996) suggested that, in fact, TBT inhibits the sulphur conjugation and excretion of testosterone, which consequently leads to the increased androgen concentration in gastropod tissues. Whichever mechanism is correct (both could be, of course), the overall effect is increased testosterone concentrations, and hence masculinization of female molluscs.

In summary, there are data available which suggest that adverse reproductive effects can be induced in wildlife by endocrine disrupting chemicals in 'natural' environments, although the majority of sites examined have been subject to

profound pollution incidents, whether they be acute or chronic. Far less information is available regarding the long-term effects of lower concentrations of such chemicals on wildlife populations.

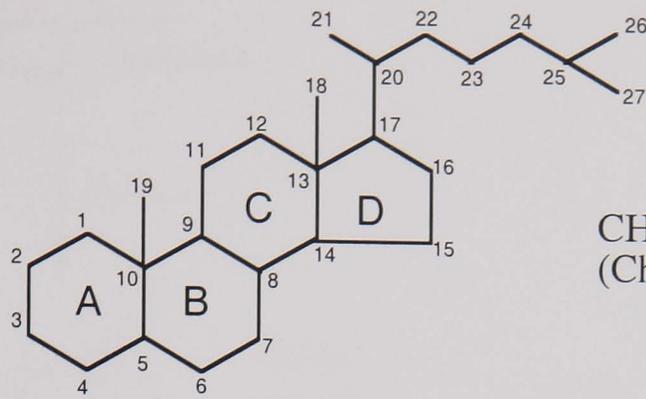
1.2 GONADAL SEX STEROIDS

1.2.1 Class, structure, and activity

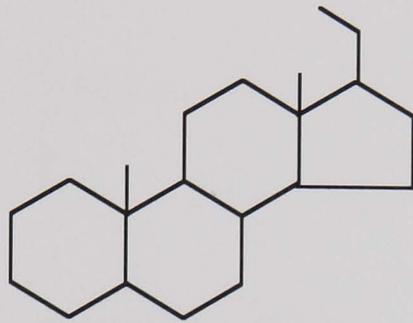
There are three main classes of sex steroids, the estrogens, androgens, and progestogens. They all have the same basic structure, consisting of four fused rings, with various oxygen and carbon substitutions conferring the properties of the individual steroids. Figure 1.1 shows this fused ring structure, along with the standard labelling of rings (A to D), and carbon positions (1 to 27), from which the steroid nomenclature is determined. Also shown here are estrane, androstane, and pregnane, which are the core structures of the estrogens, androgens, and progestogens, respectively.

The synthesis of the steroid hormones is initiated by the conversion of acetate to cholesterol. This is the precursor of all steroid hormones. Cholesterol is transformed to pregnenolone, which is itself metabolised by a sequence of enzymes to form the steroids. A scheme depicting the pathway of steroid synthesis is shown in figure 1.2. Corticosteroids are also included in this schematic, but these are not considered sex steroids, and so will not feature in the following discussion.

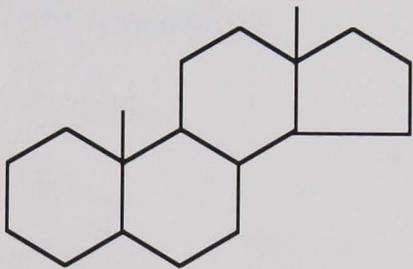
One aspect of steroid chemistry worth drawing attention to at this stage is conjugation. This involves the formation of an ester with sulphuric or glucuronic acid, thus making the steroid more soluble, together with deactivating it. In mammals, this process occurs in the liver, and is considered part of the excretory process (although some of the sulphate conjugates may be reconverted back to active compounds). In non-mammalian vertebrates, however, there may be alternative functions for these conjugated products. In the teleost testis, for example, the capacity for production of glucuronides is far higher than in the mammalian testis, and it has been suggested that since more glucuronides are



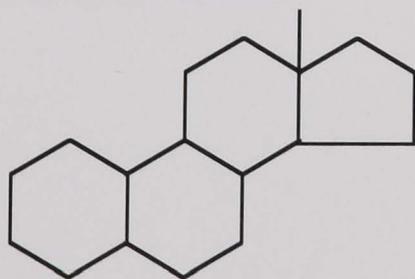
CHOLESTANE (C27)
(Cholesterol)



PREGNANE (C21)
(Progestins
e.g. Progesterone)



ANDROSTANE (C19)
(Androgens
e.g. 11-ketotestosterone)



ESTRANE (C18)
(Estrogens
e.g. 17 β -estradiol)

Figure 1.1

The core structures and root names of the steroid hormones

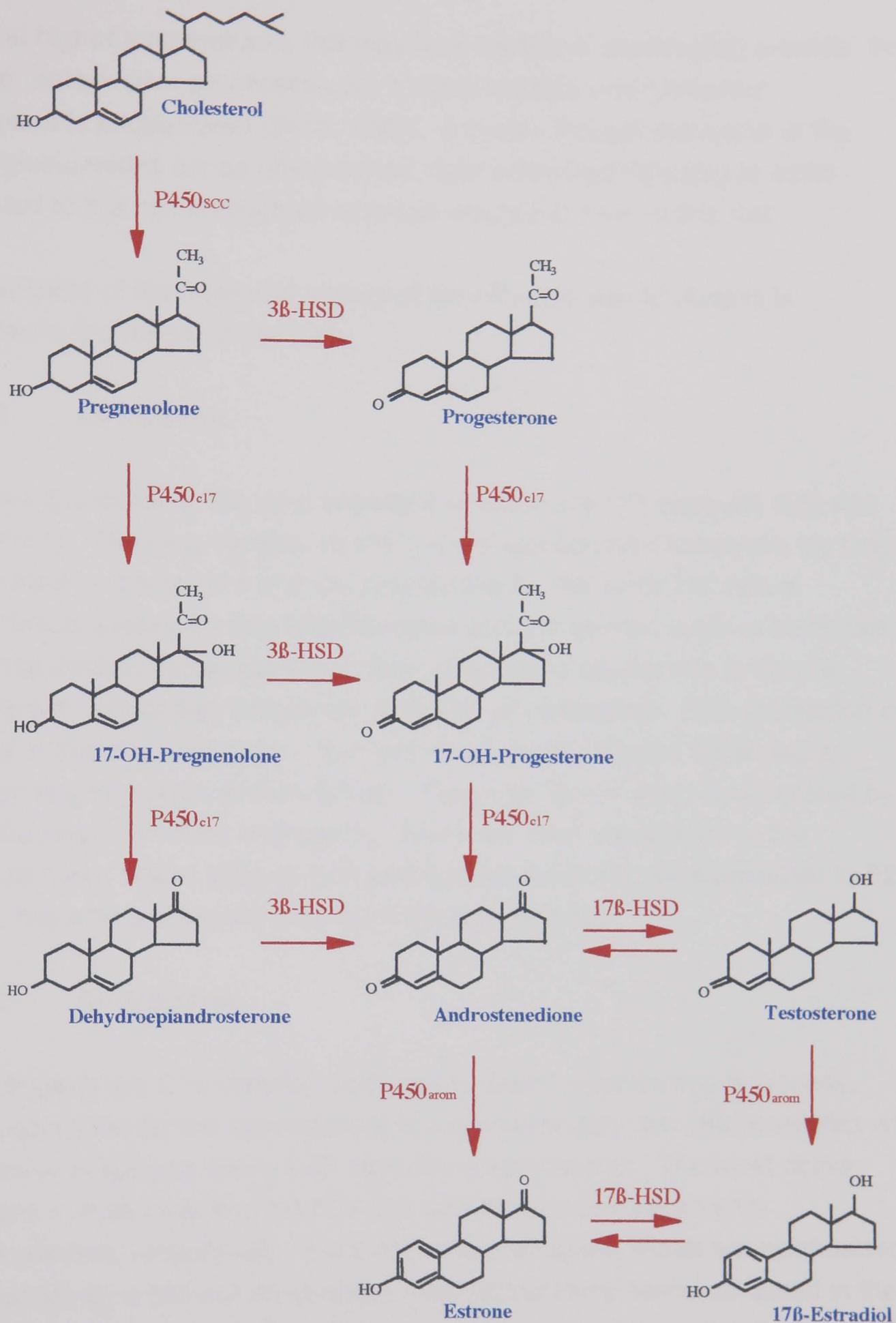


Figure 1.2

The pathway of sex steroid biosynthesis in teleosts, also illustrating the enzymes involved in conversion of steroids at each step. In males, testosterone is converted via 11β-hydroxytestosterone to 11-ketotestosterone.

formed at higher temperatures, this may be a means of deactivating steroids, thus delaying reproductive processes until a more suitable environmental temperature is encountered (Scott, 1987). It is also thought that some of the steroid glucuronides act as pheromones; their enhanced solubility in water (compared to the non-conjugated steroids) would aid them in this role.

A brief resumé of the biological activity of the different steroid classes is presented in the following sections.

1.2.1.1 Estrogens

These are C₁₈ steroids, the most important of which are 17β-estradiol (E2) and estrone (E1). They are considered the 'female' reproductive hormones, as they are secreted by the ovaries and are responsible for the control of sexual differentiation, secondary sex characteristics and the estrous cycle in mammals. In non-mammalian oviparous vertebrates, they play a crucial role in oocyte development, in that they induce the synthesis of vitellogenin. The production of estradiol in teleosts occurs via a 'two-cell' mechanism. Figure 1.3 shows a simplified diagram of an ovarian follicle. The outer 'theca' layer is stimulated by gonadotropins to produce androgens. These are then transported to the granulosa layer, where testosterone and androstenedione are aromatized to E2 and E1, respectively (via the enzyme P450 aromatase).

1.2.1.2 Androgens

The androgens are C₁₉ steroids, and are the 'male' reproductive hormones, being responsible for the development of male secondary sex characteristics and reproductive behaviour, along with stimulating spermiation. The most active androgens in mammals and teleosts are dihydrotestosterone and 11-ketotestosterone, respectively. Similarly to the estrogens, these are synthesised in the gonads by a two cell mechanism, with testosterone being produced in the Leydig cells, and metabolised to the more active compounds in the Sertoli cells. Testosterone is also present in high concentrations in the female teleost. Indeed, it is often present at levels exceeding those found in males. However, the role of testosterone in the female is unclear, although it has been suggested that it may be concerned with the maintenance of oocyte maturation processes following vitellogenesis (Kime, 1993).

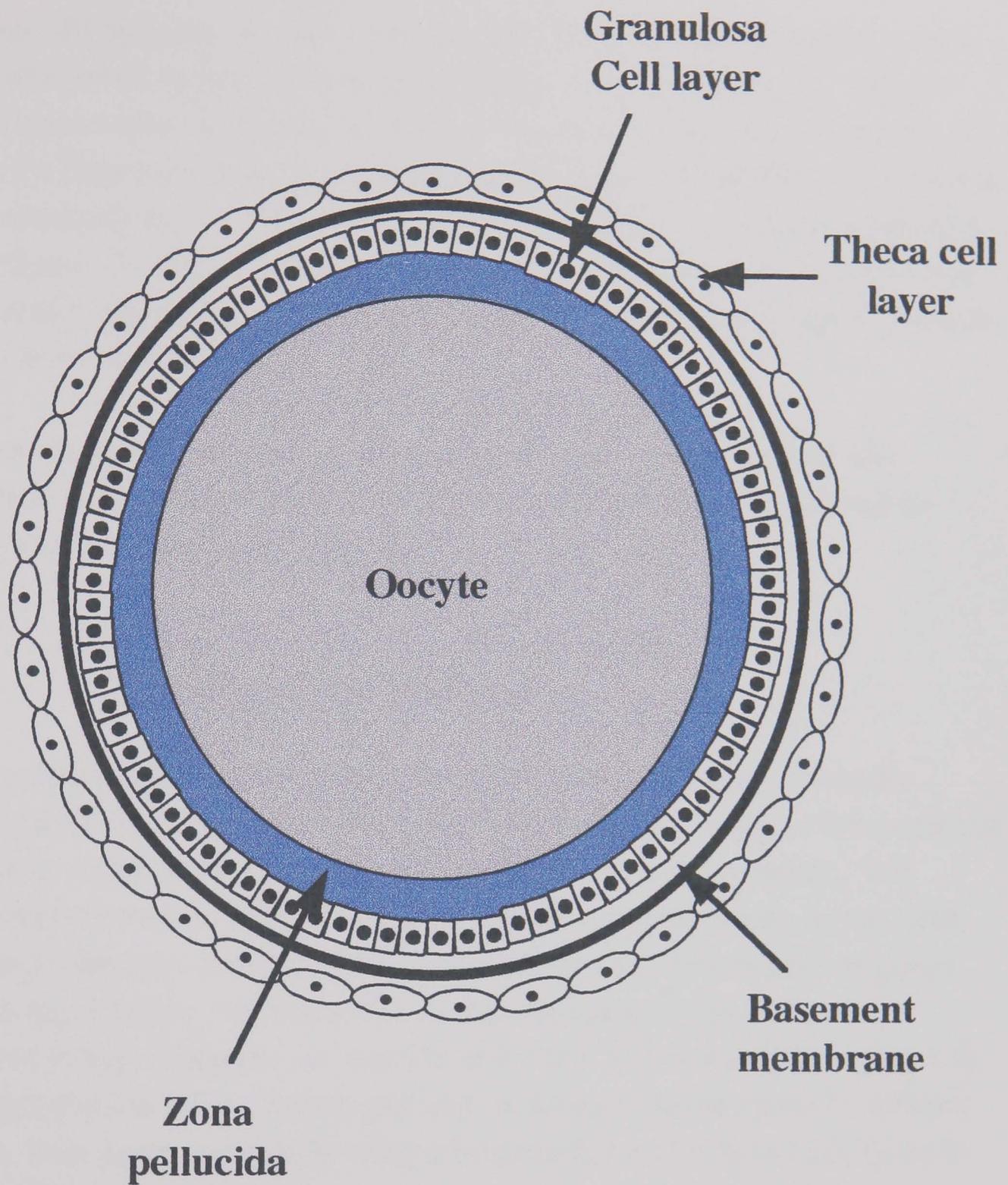


Figure 1.3

Schematic diagram of a teleost ovarian follicle, indicating the layers of the follicular wall where steroids are synthesised: androgens are produced in the theca layer, and following their move to the granulosa layer, are aromatised to estrogens.

1.2.1.3 Progesterogens

These are C21 steroids. In mammals, progesterone is produced mainly by the ovaries, with minor secretions also by the testes and adrenal glands, and is primarily responsible for the maintenance of the placenta during pregnancy. In teleosts, the nature and role of progesterogens differs, in that progesterone itself is of little consequence, but from this other progesterogens, e.g. 17,20 β -dihydroxy-4-pregnen-3-one (17,20 β -P) are synthesised, and these exert their influence over the final maturational stages of oocyte development, and also instigate germinal vesicle breakdown prior to spawning.

Endocrine disruption to date has been predominantly concerned with the estrogens and androgens and their inhibitors, and thus the focus will rest on these for the remainder of this chapter.

1.2.2 Activation of the receptor

Steroid receptors are intended to be specific for a certain ligand. Once the steroid crosses the target cell membrane, it binds to a free receptor within the cell cytoplasm or nucleus; the binding of the ligand activates the receptor. The ligand-receptor complex then attaches to a specific regulatory site on the DNA, known as a 'response element' (RE). This step initiates transcription of genes which are regulated by the respective steroid, following which mRNA is transported to the cytoplasm and translated into the relevant protein (figure 1.4). It is thought that the RE is located upstream of different target genes in different cell types, thus accounting for the contrasting response of different cell types to the same steroid.

The majority of reports regarding the estrogen receptor (ER) are concerned with what is now referred to as 'ER α '. Until recently, there was thought to be only one ER. However, in 1996, Kuiper et al reported the cloning of a second ER (referred to as 'ER β ') from the rat prostate (Kuiper et al, 1996), and subsequently an ER β has also been cloned in fish (the goldfish - Tchoudakova et al, 1999). The implications of this discovery have yet to be fully determined, although it has been shown that the specific binding of E2 to ER α and ER β is of similar magnitude (Kuiper et al, 1997), and it has also been demonstrated that

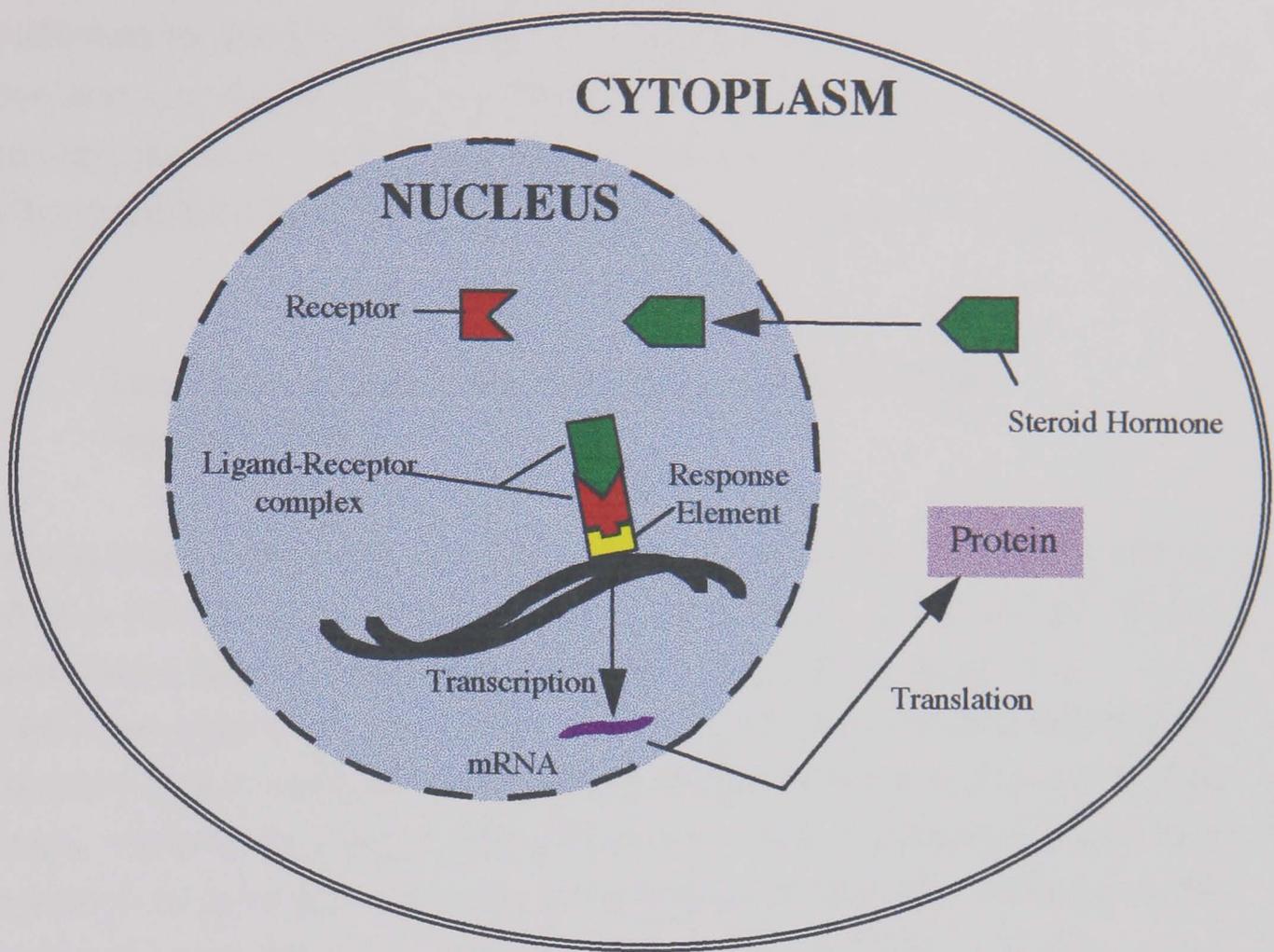


Figure 1.4

Schematic diagram illustrating the process of gene activation by steroid hormones.

xenoestrogens generally compete similarly with E2 for binding to the two receptors (Kuiper et al, 1998). However, some phytoestrogens seem to have differing affinities for the ER α and ER β (Kuiper et al, 1998), and the tissue distribution and expression of the two receptors in the rat also appears discrete. The continuing research into this aspect of endocrinology will no doubt enlighten us as to the possible differential functioning of the two receptors in the future.

1.2.3 The role of sex steroid hormones in fish reproduction

Sex determination in mammals and birds is controlled by genetic mechanisms, and in reptiles can be under the influence of temperature. In fish, the process of sex determination has not been fully elucidated, although here too, it is considered to be genetically controlled. However, the role of environmental and hormonal factors on phenotypic development is distinct in fish compared to that in mammals. Essentially, sexual differentiation in fish is considered plastic, and in the presence of estrogens the female phenotype will develop, whereas in the absence of estrogens the fish will develop male characteristics. Likewise, the presence of androgens at this early developmental stage results in the formation of the male phenotype. This situation can be artificially manipulated by the addition of pharmacological doses of hormones to the water during the labile period, just prior to sexual differentiation, which in salmonids occurs at the late pre-hatch to early juvenile stage (Piferrer and Donaldson, 1989). This is a practice undertaken by numerous commercial fish farms to produce single sex populations of fish. The resulting fish are phenotypically distinct as either males or females, and possess normal reproductive capabilities. With respect to environmental endocrine disrupters, the question is "what happens when early developmental stages are exposed to lesser concentrations of hormone mimics?" The phenotypic change may not be complete, leaving the exposed fish with intersex organs and unknown reproductive potential. This is the fundamental question currently being addressed in the freshwater fish, the roach, by Jobling and co-workers (e.g. Jobling et al, 1998) the answer to which may provide us with some understanding of the potential effect of xenobiotic endocrine disrupters on the sustainability of wildlife communities.

In maturing fish, hormones can influence secondary sex characteristics and reproductive behaviour. In addition, estrogens are essential for oocyte development in females and androgens play a key role in spermatogenesis (estrogens have also been detected in male trout, although their function is unknown). More specifically, estrogens induce the production in the liver of vitellogenin, a large glycolipophosphoprotein, which is a precursor to egg yolk proteins. It is sequestered by developing oocytes before being broken down by the enzyme Cathepsin-D to yield the primary yolk proteins. Also crucial to successful oocyte development is the production of zona radiata (zr) proteins, which assist in the development of the eggshell, the production of which is stimulated by estrogens. Vitellogenesis in annual spawners such as the salmonids follows a distinct seasonal cycle, in which E2 concentrations cycle markedly (Scott and Sumpter, 1983). This rhythm of E2 accounts for the pronounced seasonal cycle of vitellogenin, and hence ovarian development. The E2 also plays a role, via feedback mechanisms, in the concentrations of gonadotropins (FSH and LH); these hormones also play a pivotal role in oocyte development and maturation, and their functions are discussed in greater detail in Chapter 4. The responsiveness of vitellogenin to estrogenic stimulation has culminated in the measurement of this protein in blood plasma as a biomarker for the presence of environmental estrogens, particularly in males or juvenile females (which are capable of generating this protein on stimulation with estrogens, but do not normally produce it due to the low endogenous levels of circulating estrogens).

1.2.4 Occurrence of steroidal compounds in the aquatic environment

Several studies have demonstrated the presence of hormones, both synthetic and natural, in watercourses and effluents in various countries. For example, Aherne and Briggs (1989) reported levels of ethinylestradiol (EE2) of up to 15 ng/L in English rivers. Unusually, the effluents analysed in these studies were reported to contain lower concentrations of EE2 than the more dilute river water samples. However, this may have been a result of reduced recovery due to the high organic content of the effluent samples. This contrasts with the data of Stumpf et al (1996), who reported up to 62 ng EE2/L in effluents, but often were unable to detect this chemical in river water. In this case, E2 was present at

concentrations lower than EE2, of up to 20 ng/L in effluents. Ternes et al (1999) reported that EE2 was relatively persistent when in contact with activated sludge, whereas E2 was degraded. Presumably this also means that EE2 will be relatively persistent in the aquatic environment in general, and therefore may accumulate in a river which is constantly receiving contaminated sewage effluent. This may in part explain why Aherne and Briggs (1989) found higher concentrations in river water than in effluents, but does not explain why Stumpf et al (1996) were unable to detect EE2 in river water. Ternes et al (1999) also reported the important finding of the deconjugating ability of activated sludge, in that E2-glucuronides were shown to be metabolised in their experiments, releasing the active hormone. These data were supported by the observation of a vitellogenic response in male fathead minnows exposed to effluent from a simulated STW which had been dosed with E2-glucuronide. In contrast, fish administered E2-glucuronide directly, via a straightforward flow-through system, exhibited no such estrogenic response (Panter et al, 1999).

Significant amounts of natural hormones may also be present in agricultural waste, following excretion by farm animals. Shore et al (1993) detected up to 353 ng E2/L in agricultural sewage, and Nichols et al (1997) showed that E2 can be released from poultry litter, which is often applied to land as a fertiliser, therefore leading to agricultural runoff contaminated with naturally excreted hormones.

Although the estrogenic activity of sewage effluents observed in the UK has been conjectured to be in part due to estrogens such as EE2, until recently this was speculation and it was not clear whether natural and/or synthetic hormones were present at concentrations sufficient to induce the estrogenic responses in fish, such as those observed by Harries et al (1997, 1999), and also whether they are the predominant estrogenic components of such effluents or whether there might be industrial or plant-derived estrogens contributing significantly to the estrogenic activity of these effluents. To address this issue, Desbrow et al (1998) used a toxicity identification and evaluation (TIE) approach, based on using an in vitro assay for estrogens (the recombinant yeast assay - as described in section 2.1.1) to track the estrogenic activity during the fractionation of STW effluents, in an attempt to identify the main estrogenic chemicals in these effluents. The study focused on STWs receiving domestic, as opposed to industrial, waste, and found E2 and estrone (E1) to be present in each of the seven effluents studied, at

concentrations ranging from 1 to 50 ng/L and 1 to 80 ng/L, respectively. These hormones were the principal estrogenic components of these particular sewage effluents. EE2 was also detected in three out of the seven samples, albeit at lower concentrations than the natural hormones (0.2 to 7 ng/L). A subsequent 3 week laboratory study demonstrated a statistically significant vitellogenic response in male rainbow trout exposed to 100 ng E2/L, and a response which was greater than, although not significantly different from, that observed in the control fish, in fish exposed to 10 ng E2/L (Routledge et al, 1998). The authors also demonstrated that a combination of E2 and E1 induced a greater response than either hormone alone, and concluded that levels of estrogens found in the environment are capable of inducing vitellogenin production in fish. This report did not cover the response of fish to EE2, although an earlier study has suggested a no observed effect-concentration (NOEC) of 0.1 to 0.3 ng EE2/L (Sheahan et al, 1994). It must be noted, however, that Desbrow et al (1998) based their conclusions on data from domestic effluents, and that the estrogenic components of industrial effluents, such as those encountered in the river Aire, may differ, although this has not yet been established.

1.3 *NATURALLY OCCURRING XENOESTROGENS IN THE ENVIRONMENT*

Probably the largest single class of xenoestrogens to which humans, and many wildlife species also, are exposed, are the phytoestrogens. This term covers a wide range of plant-derived compounds, and includes the flavonoids, the most prolific group of phytoestrogens, of which the isoflavones (e.g. genistein, daidzen), flavones (e.g. kaempferol), flavanones (e.g. naringenin), and the chalcones (e.g. phloretin) are sub-groups. Coumestrol is one of the most potent phytoestrogens, and is also a flavonoid-derived compound. Other groups of naturally occurring chemicals produced by plants (or fungi) which possess estrogenic members include the lignans (e.g. enterodiol, enterolactone), the mycoestrogens, which are of fungal origin (e.g. zearalenone), and the plant sterols (e.g. β -sitosterol).

Since phytoestrogens are naturally occurring chemicals, humans and wildlife are exposed to them regularly via foodstuffs, as well as via their surroundings.

However, depending on the individual (or, in some cases, the population), phytoestrogen exposure of humans can be high, particularly if soy products form a major part of the diet. For example, soybeans and soyfoods have been reported to contain approximately 20,000 to 160,000 $\mu\text{g}/100\text{g}$ dry weight of the isoflavones daidzen and genistein (Mazur and Adlercreutz, 1998). In the aquatic environment too, excessive concentrations of phytoestrogens can occur. Pulp and paper mill effluents are the most frequently reported contributors to phytoestrogen pollution. For example, total sterol concentration of pulp and paper mill effluents in the US was reported to range from 71 to 535 $\mu\text{g}/\text{L}$; the most prolific plant sterol detected in this study was β -sitosterol (Cook et al, 1996). In Canada, bleached kraft mill effluent (BKME) was found to contain 1200 μg β -sitosterol/L after primary treatment, and 280 μg β -sitosterol/L following secondary treatment (MacLatchy and van der Kraak, 1995). β -sitosterol has also been detected in effluent from domestic sewage treatment works in Germany, although at far lower concentrations compared to the pulp mill effluents; concentrations reported in STW effluents, river water, and tap water were up to 402 ng/L, up to 56 ng/L, and 20 to 60 ng/L, respectively (Stumpf et al, 1996). A further possible source of sterol contamination is waste from the fat industry (Protiva et al, 1984), although data concerning concentrations in effluents from this origin are sparse, and the extent of pollution emanating from this source is unclear.

Although the phytoestrogens are weakly estrogenic in comparison to the natural estrogens such as E2 and E1, they appear to be among the most potent xenoestrogens assayed in vitro, relative to other, synthetically produced, endocrine disrupting chemicals (Routledge and Sumpter, 1996; Gaido et al, 1997; Knudsen and Pottinger, 1999). This does not necessarily mean that this significant estrogenic activity would be translated to an in vivo situation, however, when factors such as metabolism, availability, and time of exposure, might all influence the enhanced / reduced activity of these chemicals in a whole animal. There might also be other mechanisms by which these chemicals can affect the endocrine systems of animals, such as inhibition of the activities of enzymes involved in the steroidogenic pathway. For example, some flavones are thought to inhibit P450 aromatase activity (Kellis and Vickery, 1984), and some phytoestrogens (including coumestrol and genistein) have been reported to inhibit the activity of 17 β -hydroxysteroid oxidoreductase (Mäkelä et al, 1995), which catalyses the conversion of estrone to 17 β -estradiol. MacLatchy and van der Kraak (1995) also reported reduced steroid synthesis in fish injected with β -

sitosterol, which they attributed either to effects on available cholesterol levels, or on the activity of the side chain cleavage enzyme (P450_{SCC}), which may partially account for the reproductive disruption observed in fish exposed to this chemical via kraft mill effluents (discussed in section 1.1.2.5). However, not all authors agree that phytosterols are estrogenic. For example, whereas Mellanen et al (1996) reported β -sitosterol to induce estrogenic responses in vivo in rainbow trout, and in vitro in breast cancer cells, and an elevated plasma vitellogenin concentration was reported in fish exposed to this chemical by Tremblay and van der Kraak (1999), Baker et al (1999) concluded that the phytosterols were not estrogenic in either the in vitro or in vivo assays employed in their study.

Phytoestrogens have been found to be responsible for various reproductive disorders in animal populations, such as infertility in captive cheetahs fed on a soybean-based diet (Setchell et al, 1987). The best known example of adverse effects of phytoestrogens, however, is the so-called 'clover disease', which manifested itself in sheep of Western Australia way back in the 1940's (Bennetts et al, 1946). The symptoms observed ranged from changes in external and internal reproductive organs, including cervical changes which led to reduced sperm breakthrough, mammary development in unmated ewes and wethers, and behavioural changes in ewes (Adams et al, 1988; Adams, 1998). Essentially, a reduction in fertility, which impacted severely on lambing success, was the outcome. Extensive research in this area led to the conclusion that the amount of formononetin in the clover pastures was the influential factor in causing these disorders (Millington et al, 1964), with this compound being metabolised to the estrogenic chemical, equol, in the rumen of the sheep (Cox and Braden, 1974). The subsequent cultivation of clover strains with lower formononetin concentrations has led to a reduced incidence of clinical outbreaks of clover disease, although subclinical infertility can still be a problem in sheep in this area of Australia.

Despite these examples of adverse effects induced following exposure to naturally occurring phytoestrogens, it is considered by many that these compounds can actually play a protective role against certain hormone-related cancers (see Adlercreutz, 1995, for a review). Epidemiological studies have indicated (although it must be noted that they have not proven) a link between Western diets and an increased incidence of, particularly, breast cancer (Adlercreutz, 1990; Messina et al, 1994), but also prostate cancer (Adlercreutz et

al, 1993). Cell culture experiments using cancer cell lines suggest that certain phytoestrogens act as antiestrogens in that they inhibit the proliferation of these estrogen-dependent cells (Mousavi and Adlercreutz, 1993; So et al, 1996; Peterson and Barnes, 1996). In vivo experiments have also illustrated the inhibition of carcinogen-induced tumours in rats by flavonoids and citrus juices (So et al, 1996), although a recent article reported that in utero exposure of rats to genistein actually increased tumour incidence (Hilakivi-Clarke et al, 1999).

The mechanisms by which these compounds provide this apparent preventative effect are not clear, although it has been suggested that some of the phytoestrogens may stimulate the production of sex hormone binding globulin (SHBG), thus reducing the amount of available natural estrogens in the plasma (Adlercreutz, 1990; Mousavi and Adlercreutz, 1993). Whitten and Naftolin (1998) concluded that in vitro evidence suggests the process is more likely to involve receptor-mediated mechanisms than alterations to enzyme behaviour. In essence, there could be a multitude of pathways within and between particular groups of phytoestrogens by which they exert their endocrine disrupting effects, whether they are adverse or favourable.

1.4 SYNTHETIC HORMONE MIMICS IN THE ENVIRONMENT

Numerous industrial compounds have been found capable of eliciting hormone receptor-mediated responses in vitro. Many of these have also been associated with adverse reproductive effects in vivo in laboratory based studies. It is nevertheless likely that many environmental pollutants have not yet been identified as hormone mimics, and it is also possible that those which have been labelled 'environmental estrogens' can exert their effects via receptors or mechanisms other than solely the ER-mediated pathways. Some of the groups of industrial chemicals which are most widely accepted to behave as hormone mimics are discussed below. The bulk of the studies discussed are concerned with estrogenic activity as opposed to androgenic activity, and agonistic as opposed to antagonistic activity. One major reason for this focus on estrogenic chemicals is the widespread availability of both in vitro and in vivo assays for estrogens. There are currently fewer definitive endpoints available for

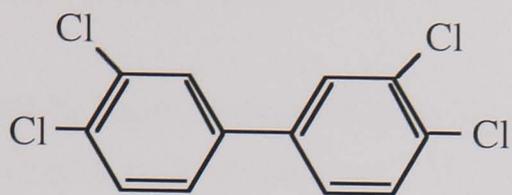
verification of androgenic and, particularly, anti-androgenic behaviour of chemicals, and hence the (possible) presence in the environment of chemicals with these activities is much less well studied.

1.4.1 Organohalogen compounds

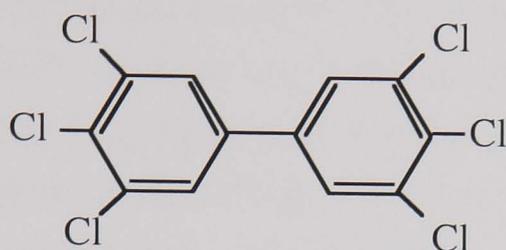
1.4.1.1 Polychlorinated biphenyls

The polychlorinated biphenyl's (PCBs) were first introduced into the commercial world in 1929, by the Monsanto Chemical Corporation, and are semi-volatile, persistent organic compounds, of which there are 209 different congeners. Some examples of PCB structures are shown in figure 1.5. Although the properties of individual congeners are dependent upon the number and positioning of the chlorine atoms, in general the PCBs are highly stable and inert, rendering them suitable for use in capacitors and transformers, as lubricants and plasticisers, as well as being employed to prevent the evaporation of pesticides following spraying. PCBs were first banned voluntarily in 1971, and legally in 1979, but are still present in closed systems of old equipment. As a result of their resistance to chemical and thermal degradation, they are still one of the most ubiquitous environmental pollutants, and have been detected in samples far from any industrial influences, such as areas of the Arctic circle (Thomas et al, 1992). Another consequence of the persistence and lipophilic nature of PCBs is the bioconcentration/biomagnification of these chemicals in organisms at various levels of the ecosystem (Evans et al, 1991). These processes lead to increased body burdens of PCBs in top predator species, e.g. Narwhal in the Canadian Arctic (Muir et al, 1992), and common seals in Northern Ireland (Mitchell and Kennedy, 1992), and also in their offspring. In mammals, for example, transfer of PCBs from mothers to young has been demonstrated to follow both uterine (Darnerud et al, 1996) and lactational (Sinjari et al, 1996) pathways, thus increasing the risk of exposure of young at sensitive developmental stages to highly toxic chemicals. An equivalent exposure situation may be encountered in non-mammalian vertebrates, whereby accumulation of PCBs has been observed in the ovaries of Atlantic croaker (Ungerer and Thomas, 1996), which may be due to the attraction of these lipophilic compounds to vitellogenin. Accumulation of such chemicals in the ovaries will, presumably, lead to deposition in eggs, and

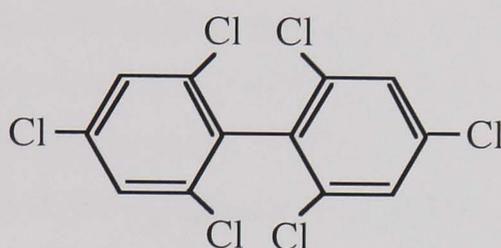
PCBs



3,3',4,4'-tetrachlorobiphenyl
(coplanar)

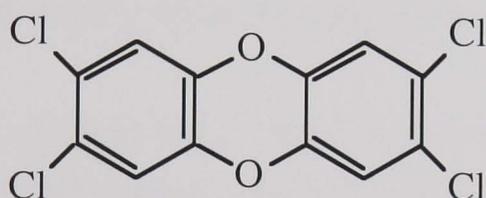


3,3',4,4',5,5'-hexachlorobiphenyl
(coplanar)



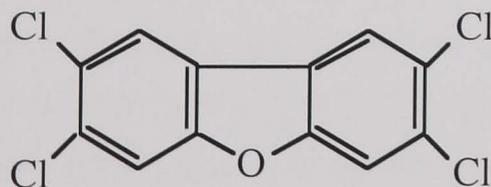
2,2',4,4',6,6'-hexachlorobiphenyl
(non-coplanar)

PCDDs



2,3,7,8-tetra-chlorodibenzodioxin
(TCDD)

PCDFs



2,3,7,8-tetra-chlorodibenzofuran
(TCDF)

Figure 1.5

The structures of some of the PCBs, depicting examples of both coplanar and non-coplanar forms, together with examples of dioxins and furans.

hence exposure of larvae. PCBs have also been detected in the eggs of gulls (Pastor et al, 1995a, 1995b).

With respect to their hormonal activity, it appears that PCBs exert their effects through numerous mechanisms. Various PCB congeners have been demonstrated to induce estrogenic or anti-estrogenic effects in both in vitro and in vivo assays (Jansen et al, 1993; Nesaretnum et al, 1996; Nesaretnum and Darbre, 1997). Soto et al (1995) assayed 18 different congeners for estrogenic activity in the E-Screen, and concluded that out of these, 5 were estrogenic, none of which were coplanar (i.e. all of the estrogenic PCBs had substitutions at one or more ortho positions). Nonetheless, coplanar congeners can disrupt endocrine functions via alternative pathways (Safe et al, 1985). Interaction with the aryl hydrocarbon receptor (AhR) is considered one of the key mechanisms of action of the PCBs (Safe et al, 1985; Krishnan and Safe, 1993), together with the induction of the P₄₅₀ group of enzymes, which are responsible for metabolism of foreign chemicals. This action in itself may enhance the estrogenic activity of the PCBs, since their hydroxylated metabolites have been reported to be more active than the parent compounds (Soto et al, 1995), thus in effect creating a cyclic 'positive feedback' type of response.

Laboratory studies have established the ability of aroclors (industrial mixtures of PCB's) to affect reproductive development of rats (Gellert, 1978) and to alter the sex ratio and gonadal development of rainbow trout (Matta et al, 1998). Individual congeners have been reported to suppress maturation of female white perch (Monosson et al, 1994), and to reverse the temperature-dependent sex determination of turtles (Bergeron et al, 1994). A link with reproductive dysfunction in wild populations is more circumstantial; nonetheless, Murk et al (1996) observed a relationship between various reproductive endpoints (such as egg size and incubation period) and yolksac PCB content in a species of bird, the tern. Likewise, a correlation between whole egg PCB concentration and breeding success of cormorants in the Netherlands was reported by Dirksen et al (1995). Much research into PCB-contaminated freshwater sites has focused on the Great Lakes in the USA/Canada. Here, significant correlations have been found between PCB concentration and hatching success of lake trout inhabiting these waters. Knock-on effects have also been noted in fish-eating bird species in this area (Gilbertson et al, 1991).

The mechanisms by which PCB's induce adverse reproductive effects in wildlife are certainly complex, but that such effects occur is unquestionable.

1.4.1.2 Dioxins and furans

The polychlorinated dibenzo-p-dioxins (PCDD's) and polychlorinated dibenzo-p-furans (PCDF's) are similar in structure to the PCB's (see figure 1.5), and, like the PCB's, they are ubiquitous and persistent in the modern environment. However, they are not deliberately manufactured, but are by-products of incineration and manufacturing procedures. Dioxins have been detected at trace concentrations (parts per billion to parts per trillion) in environmental samples such as sewage (Hagenmaier et al, 1992), in fish exposed to Kraft Mill effluent (Owens et al, 1994), in samples taken from landfill sites (Hansen and O'Keefe, 1996), and in drinking water (Lampi et al, 1990). Their bioaccumulation factors in fish have been reported as ranging from 3000 to 106,000 (Frakes et al, 1993), indicating the absence of metabolic potential of such organisms for these chemicals. The most toxic dioxin is 2,3,7,8-tetrachlorodibenzo-p-dioxin (frequently referred to as 'TCDD'). Other related compounds are often given potency values relative to this chemical; these are known as Toxic Equivalency Factors (TEF's) (Safe et al, 1996).

The dioxins and furans are essentially 'endocrine disrupters' rather than straightforward hormone mimics, since they can act on multiple elements of the endocrine system (Birnbaum, 1994). It has been known for several years that TCDD can cause infertility in rats (Gray and Kelce, 1996). Reproductive effects in male rodents exposed in utero to TCDD include delayed puberty, decreased sperm counts and epididymal sperm storage (Gray et al, 1995); in females adverse effects include delayed puberty, decreased ovarian weight, and decreased fecundity (Gray and Ostby, 1995). TCDD has also been reported to inhibit estrone-induced implantation in hypophysectomised adult female rats (Johnson et al, 1992). This chemical also caused diminished egg numbers and delayed oocyte development when administered to adult zebrafish (Wannemacher et al, 1992).

A review of the array of anti-estrogenic responses observed in organisms exposed to TCDD, along with activities of this chemical in in vitro systems, can be found in Safe et al (1991). However, it is thought that these effects are not

directly ER-mediated, but that these compounds are AhR ligands (Safe et al, 1991), although the precise mechanism behind the anti-estrogenic responses subsequent to the dioxins binding to the AhR are as yet unknown.

1.4.2 Pesticides

The potential for exposure of wildlife in general (and even humans) to pesticides may be higher than for many other synthetic chemicals, since these compounds are deliberately and prolifically released into the open environment, and applied directly to food crops throughout the world. The most prolific and toxic pesticides are of organochlorine structure, and along with their harmful nature, are lipophilic and therefore persistent in the environment and biomagnify up the food chain. Many of these organochlorine pesticides have been banned in developed countries (although they still persist in environmental samples), but may still be used in the developing world, where serious health issues such as malaria and other insect borne diseases are a major priority. Pesticides found capable of mimicking natural hormones (either in vitro or in vivo) include DDT, methoxychlor, endosulfan, dieldrin, lindane, kepone (chlordecane), and toxaphene (Soto et al, 1995; Petit et al, 1997). Vinclozolin (a fungicide) has also been implicated as an endocrine disrupter (Kelce et al, 1994).

One of the most widely researched organochlorine pesticides is DDT (dichlorodiphenyltrichloroethane). The commercial DDT mixture comprises different isomers, such as o,p'-DDT (15-20%), and p,p'-DDT (the predominant isomer). These compounds degrade to DDE and DDD, which may themselves possess hormonal characteristics. The presence of DDT in the environment has been associated with numerous adverse reproductive effects, primarily in wildlife, but also in humans. Although in vivo laboratory studies conducted as long ago as 1969 indicated the estrogenic nature of o,p'-DDT (Bitman et al, 1969), reports of effects observed in exposed wildlife date back to the 1980's, where behavioural anomalies and skewed sex-ratio of gulls could be correlated to organochlorine pollution (Fry et al, 1987), and DDT injected into eggs at concentrations similar to those found in wild birds eggs led to feminisation of the gonads in male chicks (Fry and Toone, 1981). Aquatic species have also been observed to possess reproductive abnormalities associated with DDT contamination. For example, Hose et al (1989) reported an exposed population

of white croaker to be exhibiting decreased fecundity and fertility, and delayed oocyte development compared to those sampled from a reference site; significantly decreased testosterone levels were measured in porpoises found to possess high levels of the DDT metabolite, DDE (Subramanian et al, 1987); and a possible link between decreased penis size, along with lowered plasma testosterone concentrations, and elevated levels of p,p'-DDE in alligators inhabiting Lake Apopka was suggested by Guillette et al (1996). With respect to effects in humans, Wolff et al (1993) surveyed breast cancer patients and found that mean levels of DDE in such patients were significantly higher than in controls. However, subsequent studies have found no positive correlation between DDT levels and the risk of breast cancer (Safe, 1997).

The endocrine activity of DDT and its metabolites in vitro is not straightforward. For example, o,p'-DDT is generally considered an estrogen receptor agonist, and has been reported as being estrogenically active in various in vitro assays (Donohoe and Curtis, 1996; Kelce et al, 1995; Vonier et al, 1996), but this isomer has also been found to be capable of binding to the AR, albeit with a lower affinity than to the ER (Kelce et al, 1995). On the other hand, the major metabolite of DDT in humans, p,p'-DDE, has been found to be a relatively potent anti-androgen (being approximately equipotent to hydroxyflutamide in inhibiting androgen-induced AR transcriptional activity) but also interacts (more weakly) with the ER (Kelce et al, 1995). This situation, where a chemical can bind to more than one steroid receptor, may be quite common (Sohoni and Sumpter, 1998). It would be unwise to try to predict from the results of these assays which pathway would generate the overriding effect in vivo. This illustrates the necessity for in vivo tests in this area of research, since metabolites might possess endocrine activity as well as their parent compounds, in addition to the possibility that these chemicals (both parent compounds and metabolites) may have multiple mechanisms of action.

Another pesticide which may give misleading results if the parent compound alone is assessed for endocrine activity is methoxychlor. This was introduced as a DDT substitute, but has been demonstrated to induce estrogen-like responses in vivo (Welch et al, 1969; Bitman and Cecil, 1970; Kupfer, 1975; Gray et al, 1988). However, the parent compound is not itself an ER agonist (Nelson, 1974; Bulger et al, 1978a; Bulger et al, 1978b). On investigation, Ousterhout et al (1981) discovered that the major metabolites of methoxychlor, these being the

mono- and bis-phenol derivatives, possessed significant estrogenic activity in an in vitro receptor binding study. There are two important messages from these data; on the one hand they warn against assessing the parent compound alone in vitro, since potential adverse effects of its derivatives may be overlooked and the chemical may incorrectly appear benign; on the other, these metabolites are more water soluble and therefore less likely to bioaccumulate than methoxychlor itself, and hence concentrations may not reach levels at which adverse effects would be manifest.

Lindane is a pesticide which is most often available in its refined form (γ -HCH), but which is sometimes produced as a commercial mixture, consisting of 3 main isomers, namely γ -HCH, which confers the required insecticidal properties, α -HCH, the principal isomer at 60-70%, and a contaminant, β -HCH, which can be present in the mixture at 5-10%. The latter isomer is that which is considered an estrogen mimic, and is capable of inducing vitellogenin production in the guppy (Wester et al, 1985), and testis ova in male medaka (Wester and Canton, 1986). However, γ -HCH has also been demonstrated to disrupt reproductive processes in the zebrafish (Braunbeck et al, 1990), and it affected steroid production in an in vitro study employing roach testes (Singh and Kime, 1995). More recently, lindane (γ -HCH) was reported to decrease fecundity in mink exposed to the pesticide over 3 generations (Beard et al, 1997).

Results from the 'E-Screen', which employs an MCF-7 cell line to assess estrogenicity of chemicals, led Soto et al (1994) to conclude that each of the pesticides endosulfan, dieldrin, and toxaphene were weakly estrogenic. Endosulfan was observed to affect the reproductive behaviour of tropical fish, and delayed egg laying by the females, in a study performed by Matthiessen and Logan (1984). Although these effects did not definitively occur via an endocrine-mediated pathway, mating behaviour can nonetheless influence survival of a population, and should not be overlooked.

Another pesticide known to be estrogenic to both birds and mammals is kepone, also known as chlordecane (Palmiter and Mulvihill, 1978; Hammond et al, 1979; Eroschenko and Palmiter, 1980), with decreased testicular and epididymal weight observed in hamsters administered this compound (Gray, 1982).

Vinclozolin is a fungicide which is not as persistent as some of the organochlorine pesticides, but is metabolised to products (referred to as M1 and

M2) which exhibit anti-androgenic activity (Kelce et al, 1994), and gestational exposure of rats to the parent compound can generate various malformations in male offspring, with an overall effect of inhibition of sex differentiation (Gray et al, 1993).

In general it must be noted that the use of many of the above-mentioned chemicals has diminished greatly, or even ceased, in the Western world. Nonetheless, due to their persistence, the fact that they are still in use in many developing countries, and their ability for biomagnification up the food chain, many of them still have the potential to reach concentrations in top predators which may affect their capacity to reproduce, particularly if more than one such endocrine disrupting chemical is present at once. From a human viewpoint, however, and certainly from that of a Westerner, exposure to organochlorine pesticides has been put into perspective by Gaido et al (1998), when they reported that a glass of wine possessed a higher estrogen equivalency rating than the daily consumption of estrogenic organochlorine pesticides via food, indicating that natural sources of estrogens may play a larger role in dietary estrogen intake than that played by pesticides. Clearly, however, this may not be the case in countries which continue to heavily employ such toxic compounds.

Although not 'organochlorine' pesticides, the pyrethroids are perhaps more relevant to the endocrine-disrupting issue in the modern environment. These pesticides are in current, widespread usage throughout the world, and are used as ingredients in both agricultural and household products. A number of these pyrethroids, along with some of their derivatives (they are generally metabolised with relative rapidity in mammals, so the latter chemicals may be of high relevance with respect to their toxicity) were assessed for agonistic and antagonistic activity in recombinant yeast estrogen and androgen assays (Tyler et al, 2000). Several of the parent compounds exhibited affinity for one or other of the receptors. Three metabolites of permethrin were also screened, of which one exhibited both estrogenic and anti-androgenic activity, and two were estrogen receptor antagonists. Data describing the possible endocrine disrupting behaviour of these pesticides in vivo is sparse, although they have been demonstrated to reduce fertility in rats administered high doses (Wauchope et al, 1992).

1.4.3 Surfactants

The principal class of surfactants known to be capable of inducing endocrine-mediated responses in wildlife (primarily fish) are the alkylphenol polyethoxylates, or more specifically, their degradation products, the alkylphenols.

Briefly, although the estrogenic activity of the alkylphenols was reported as far back as 1938 (Dodds and Lawson, 1938), it appears that this information was not considered in the context of environmental pollution until Soto et al (1991) found nonylphenol (4-NP), which was leaching from laboratory equipment, to induce an estrogenic response in an MCF-7 cell line. Subsequent to this observation, numerous articles have identified the alkylphenols (the most common of which are octylphenol and nonylphenol) to be estrogenic in vitro (Jobling and Sumpter, 1993; White et al, 1994; Routledge and Sumpter, 1996; Celius et al, 1999; Coldham et al, 1997; Gaido et al, 1997), and in vivo (Jobling et al, 1996; Lech et al, 1996; Christiansen et al, 1999; Pedersen et al, 1999; Yadetie et al, 1999; Arukwe et al, 1997; Milligan et al, 1998).

Concern over the potential toxicity of these compounds as a result of their endocrine activity has culminated in the phasing out of the alkylphenol ethoxylates in domestic applications (e.g. ENDS, 1997), and more recently, there have been moves toward a more widespread ban covering industrial and agricultural applications also (ENDS, 1999a).

A more comprehensive discussion of the alkylphenols can be found in chapter 4 (section 4.1.1).

1.4.4 Plasticisers and other plastics components

The phthalates are one of the most widely employed classes of endocrine-disrupting compounds in current usage. Once again, a more comprehensive discussion of their occurrence in the general environment, and hormone-mimicking behaviour, is provided in chapter 3. In summary, these chemicals should not be considered collectively as 'estrogen mimics'. Some of the phthalates show weak estrogenic activity in in vitro assays (Jobling et al, 1995;

Soto et al, 1995; Petit et al, 1997; Coldham et al, 1997; Harris et al, 1997; Zacharewski et al, 1998), but some are inactive in these assays. So far, however, despite numerous reports indicating the reproductive toxicity of certain phthalates, studies demonstrating specific endocrine-mediated responses of these chemicals have proven inconclusive. More recently, the possibility that some of the phthalates may act as anti-androgens has been raised (Sohoni and Sumpter, 1998; Mylchreest et al, 1998), although the mechanisms involved in the induction of the effects observed by Mylchreest et al have not yet been elucidated (Mylchreest et al, 1999).

Another chemical frequently used as an ingredient in plastic formulations, most prolifically as a monomer for polycarbonates, but also in epoxy resins, and which has been detected in leachate from dental resins (Olea et al, 1996) and lacquer coatings in food cans (Brotons et al, 1995), is Bisphenol-A (BPA). As with the phthalates, hundreds of thousands of tonnes of BPA are produced per annum in Europe alone.

Similar to the scenario with NP, the estrogenic activity of BPA was stumbled upon when it leached from laboratory equipment (polycarbonate flasks) into medium which subsequently induced proliferation of MCF-7 cells in an estrogen-dependent manner (Krishnan et al, 1993). It was subsequently reported as being approximately 1000 times less potent than E2 in a recombinant yeast estrogen assay (Routledge and Sumpter, 1996). Primary hepatocytes of fish incubated in a medium containing BPA produced zona radiata proteins and/or vitellogenin, both of which are estrogen-dependent responses (Sumpter and Jobling, 1995; Celius et al, 1999; Smeets et al, 1999a, 1999b). Further studies with breast cancer cell lines have also confirmed the mitogenic properties of this chemical (Soto et al, 1995; Schafer et al, 1999). The effect of the presence of serum on the in vitro potency of BPA was investigated by Nagel et al (1997), and it was demonstrated that serum enhanced the potency of this chemical relative to E2, and also to OP, in MCF-7 cell lines. These results were also reflected in vivo, whereby BPA appeared to affect reproductive development in exposed rats at lower doses than OP (Nagel et al, 1997). Induction of VTG mRNA in primary hepatocytes of male amphibia, *Xenopus laevis*, was enhanced by the presence of BPA, and in the same species in vivo administration of this chemical during larval development altered the sex ratio of treated groups relative to the controls (with increased numbers of females ensuing) (Kloas et al, 1999). In fish also,

BPA has been demonstrated to induce VTG production in rainbow trout (Christiansen et al, 1998). In contrast, Nagao et al (1999) reported normal reproductive development of rats treated postnatally with BPA. It may be that the timing of exposure is the crucial factor in inducing reproductive abnormalities in such experiments, although too few studies have been published to state this conclusively.

1.5 AIMS

The aims of this project were essentially two-fold. The first was to investigate the potential estrogenic activity of individual phthalates. Prior to this study, there were very limited data available on the endocrine activities of these chemicals (e.g. Jobling et al, 1995), and their very extensive use led to this work being considered a high priority. The second aim was to determine if endocrine active chemicals could exert effects at higher centres of the axis controlling reproduction (specifically, the brain); that is, extend the findings that endocrine disrupters can affect the gonad and liver (at least of oviparous vertebrates) to determine whether they can also affect higher levels of the brain-pituitary-gonadal (BPG) axis.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 METHODS EMPLOYED IN THE ASSESSMENT OF THE ENDOCRINE DISRUPTING POTENTIAL OF CHEMICALS

Methods of determining the endocrine activities of chemicals can essentially be split into two categories: *in vitro* and *in vivo*. Each has its own distinct advantages; for example, *in vitro* assays are quicker, more economical, and generally highly specific, whereas *in vivo* assays provide us with a more realistic idea of how the chemical may behave in a whole animal, by taking into account factors such as metabolism, which may create or negate the activity of a chemical.

Opinions on the benefits of available *in vitro* assays vary (Zacharewski, 1997). Probably the best approach would be to employ a battery of different *in vitro* assays, followed by confirmation of any positive results using *in vivo* assays (Gray, 1998). It must be noted that it is also important to include in *in vitro* tests metabolites of chemicals which are known to be found in the environment or in wildlife or humans, in an attempt to avoid “false negative” responses.

In vitro assays for EDCs currently in use include mammalian cell lines, for example breast cancer cell lines, which are stimulated to proliferate by estrogens, or prostate cancer cell lines which respond to androgens; recombinant yeasts, which have been genetically modified to incorporate a gene for a steroid receptor (either estrogen or androgen); receptor binding assays, which are based on the competition between a radiolabelled ligand and an unlabelled ligand for binding to a steroid receptor; and the less frequently used fish hepatocyte cultures, which produce the egg yolk protein vitellogenin in response to estrogenic chemicals.

The most widely employed mammalian *in vivo* assay with a specifically estrogen-regulated endpoint is the rodent uterotrophic assay. In this assay, immature female rats are administered the test chemical, and their uteri weighed 3-5 days later. An estrogenic substance stimulates an increase in weight of the uterus (Hisaw, 1959). This assay is also useful in that it can be used to detect anti-estrogens (Wakeling and Valcaccia, 1983). However, despite providing more realistic data with respect to the behaviour of chemicals in a whole animal, the uterotrophic assay is nonetheless short-term, and may not be sensitive enough to

detect weakly estrogenic chemicals (Zacharewski, 1998). In fish, the induction of vitellogenin production is one of the most sensitive and easily assessed indicators of estrogenic exposure (Sumpter and Jobling, 1995). This is especially true in male or juvenile female fish, since endogenous levels of this protein are extremely low. In this thesis, a variety of in vitro and in vivo assays were used to detect and quantify the endocrine disrupting potential of chemicals. The basic methodology employed in these assays is described below; in subsequent chapters, specific details relevant to the particular objective being investigated are included.

2.1.1 Recombinant yeast assays

The recombinant yeast screen was the most extensively utilised assay for detection of endocrine activity in this thesis. This is in part due to its straightforward procedure, whereby the assay is performed in 96-well microtitre plates, thus allowing a wide range of concentrations of a test chemical to be tested in each assay; it is also both time- and cost-efficient; finally, the assay is highly reproducible, specific, and sensitive (Routledge and Sumpter, 1996).

Two different strains of recombinant yeast were used in the studies performed in this thesis, one of which contained the hER α gene, and the other the hAR gene. These strains were developed by Glaxo-Wellcome (Purvis et al, 1991) and the assays were later validated by Routledge and Sumpter, 1996 (the estrogen assay), and Sohoni and Sumpter, 1998 (the androgen assay). Essentially, the hER gene (now known as ER α , to distinguish it from the more recently discovered ER β), or the hAR gene, have been integrated into the main genome of the yeast. The yeasts also contain an expression plasmid, consisting of a response element (RE), a promoter gene, and the Lac-Z gene - which codes for the enzyme β -galactosidase. The hER (or hAR) is expressed in a form capable of binding to the RE; once activated by a bound ligand, expression of Lac-Z is triggered, resulting in the production of β -galactosidase. This enzyme is a hybrid form, possessing a peptide leader sequence, enabling it to pass across the cell wall of the yeast into the surrounding medium, where it metabolises the chromogenic substance CPRG (chlorophenol-red- β -D-galactopyranoside), causing a colour change from yellow to red. This colour change can be

monitored by eye, or measured quantitatively using a spectrophotometer. A simplified diagram of the yeast make-up is shown in figure 2.1.

2.1.1.1 Preparation and storage of medium components

All glassware was thoroughly solvent washed before use. Any glassware which had come into contact with steroids or known xenobiotic endocrine disrupters was first rinsed in 20% HCl.

The minimal medium was prepared by adding 13.61 g KH_2PO_4 , 1.98 g $(\text{NH}_4)_2\text{SO}_4$, 4.2 g KOH pellets, 0.2 g MgSO_4 , 1 ml $\text{Fe}_2(\text{SO}_4)_3$ solution (40 mg/50 ml H_2O), 50 mg L-leucine, 50 mg L-histidine, 50 mg adenine, 20 mg L-arginine-HCl, 20 mg L-methionine, 30 mg L-tyrosine, 30 mg L-isoleucine, 30 mg L-lysine-HCl, 25 mg L-phenylalanine, 100 mg L-glutamic acid, 150 mg L-valine, and 375 mg L-serine to 1 litre double-distilled water. The solution was aliquotted into 45 ml portions, sterilised at 121°C for 10 minutes, and stored at room temperature.

Vitamin solution was prepared by adding 8 mg thiamine, 8 mg pyridoxine, 8 mg pantothenic acid, 40 mg inositol, and 20 ml biotin solution (2 mg/100 ml H_2O) to 180 ml double distilled water. The solution was sterilised by filtering through a 0.2 μm pore size disposable filter, into sterile bottles in 10 ml aliquots. The solution was stored at 4°C.

A 20% w/v glucose solution was prepared, which was sterilised at 121°C for 10 minutes, and stored at room temperature.

Solutions of 4 mg/ml L-aspartic acid, and 24 mg/ml L-threonine were made, also sterilised at 121°C for 10 minutes, and stored at room temperature.

A copper (II) sulphate solution (20 mM) was filter sterilised through a 0.2 μm pore-size filter into sterile glass bottles and stored at room temperature. A solution of 10 mg/ml chlorophenol-red- β -D-galactopyranoside (CPRG) was sterilised in the same way, and stored at 4°C.

Growth medium consisted of 45 ml minimal medium, 5 ml glucose solution, 1.25 ml L-aspartic acid solution, 0.5 ml vitamin solution, 0.4 ml L-threonine solution, and 125 μl copper (II) sulphate solution.

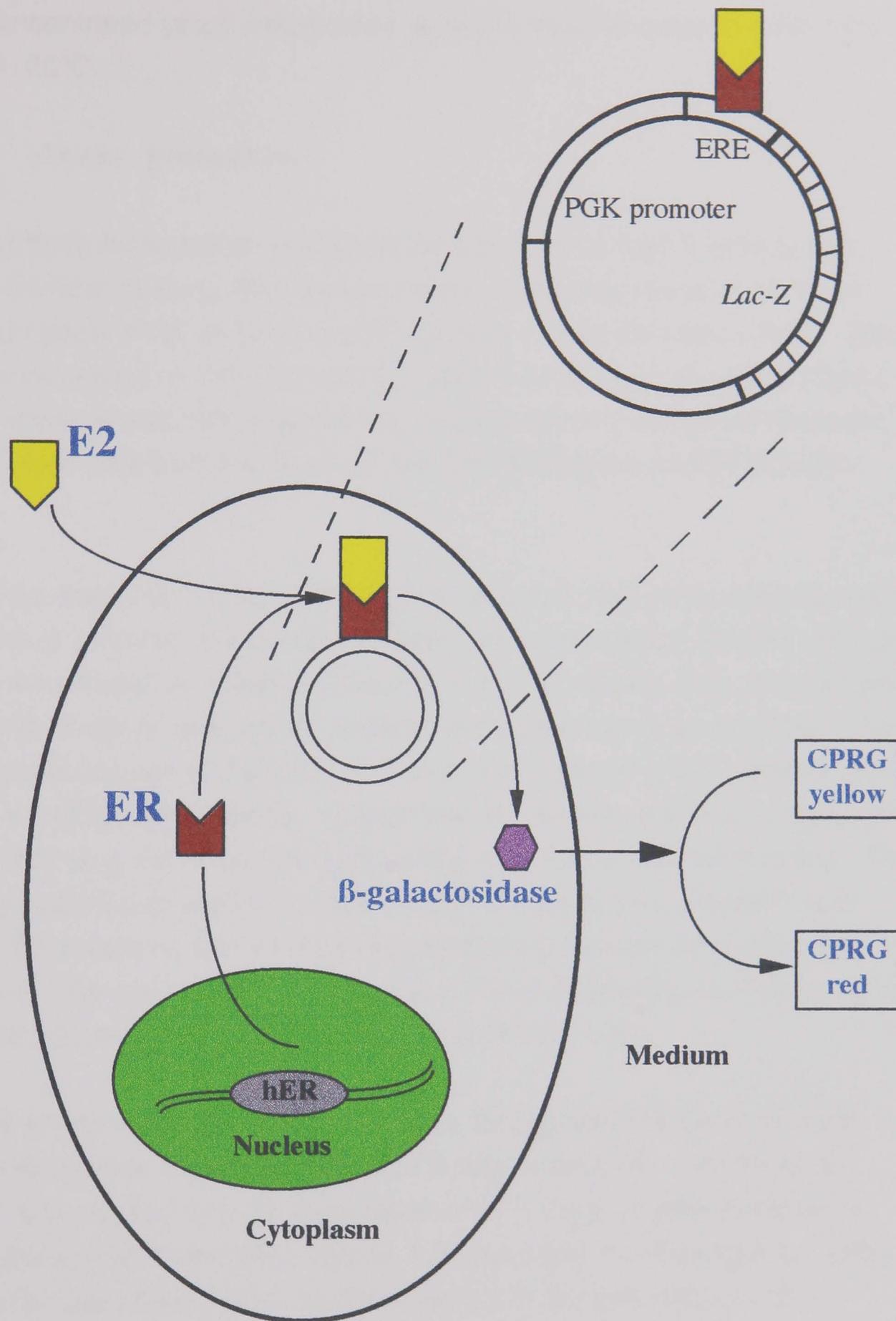


Figure 2.1

Schematic diagram of the estrogen inducible expression system in yeast. Activation of the estrogen receptor, by binding of ligand, enables the complex to bind to estrogen response elements (ERE) on the expression plasmid. This causes expression of the reporter gene Lac-Z, which produces the enzyme β -galactosidase. This enzyme passes into the medium, and metabolises the chromogenic substrate, CPRG, into a red substance. (Adapted from Routledge and Sumpter, 1996).

10 times concentrated yeast stocks were stored in minimal medium (with 15% glycerol) at -20°C.

2.1.1.2 Assay procedure

All work involving recombinant yeast was carried out in a type II laminar flow cabinet. A 24 hour yeast culture was prepared, by adding 125 µl of 10 times concentrated yeast stock to 50 ml growth medium in a sterile conical flask. The culture was incubated at 28°C for approximately 24 hours on an orbital shaker. The assay medium was then prepared by seeding 50 ml fresh growth medium with 4×10^7 yeast cells from the 24 hr culture, and adding 0.5 ml CPRG to the medium.

Chemicals were serially diluted in the solvent in which they were stored (usually ethanol). 10 µl volumes of each dilution were transferred into a 96 well optically flat bottom microtitre plate (Linbro/Titertek, ICN FLOW, Bucks, UK). 10 µl ethanol (or appropriate solvent) was added to blank wells (one row in each plate). The ethanol was evaporated to dryness. 200 µl of assay medium was added to each well using a multichannel pipette. At least one standard curve of an appropriate positive control (e.g. E2 in the estrogen assay) was included in each assay. The plates were sealed with autoclave tape (to prevent excessive evaporation of water from the medium), and shaken vigorously for 2 minutes on a microtitre plate shaker. The plates were incubated at 32°C in a naturally ventilated oven (WTB binder, BD-series, Jencons Scientific Ltd., Beds, UK).

Plates were shaken after 24 hours incubation, to disperse the growing yeast cells, and absorbance monitored after 3 to 6 days incubation. Absorbance readings at 540 nm and 620 nm were taken after 3 days incubation (using a Titertek Multiskan MCC/340 plate reader, Life Sciences Int., Basingstoke, UK) after which the plates were removed from the incubator and left at room temperature. Assays conducted using weakly estrogenic chemicals (such as the phthalates) were monitored over the subsequent days, and absorbance readings were taken once more 6 days after the assay had been set up. Assays set up using the androgen responsive yeast were incubated for 24 hours only at 32°C, after which they were incubated at 28°C for a further 24 hours, then removed from the incubator and left at room temperature until the plates were read (3 days after setting up the assay).

The absorbance readings at 540 nm (which measures the redness in the medium) were corrected for turbidity (absorbance at 620 nm) using the following calculation:

Corrected value = chemical absorbance (540 nm) - [chemical absorbance (620 nm) - blank absorbance (620 nm)].

2.1.1.3 Specificity and sensitivity

The yeast estrogen assay is highly specific for estrogens; androgens, progesterones and corticosteroids are either completely inactive or very weakly active at very high concentrations. The activities of some of these steroids in the estrogen screen are shown in figure 2.2 (adapted from Routledge and Sumpter, 1996). E2 was assayed at concentrations ranging from 1×10^{-8} M to 4.8×10^{-12} M. A concentration as low as 2 ng/L (7.35×10^{-12} M) induced a detectable increase in β -galactosidase production after a 3-day incubation period.

In contrast, the androgen screen, validated by Sohoni and Sumpter (1998), is both less specific and less sensitive. The latter observation is mainly due to the fact that it was developed earlier than the estrogen screen, and sensitivity of the assay had not been optimised. Figure 2.3 (as published in Sohoni and Sumpter, 1998) illustrates the activities of steroids in the androgen screen. DHT (dihydrotestosterone) and testosterone were approximately equipotent, the minimum detectable concentrations were approximately 4×10^{-10} M (Sohoni and Sumpter 1998). In this assay, however, E2 appeared to be able to cross-react with the androgen receptor, and was approximately 15-fold less potent than DHT and testosterone (put another way, E2 had approximately 6% of the binding potential of these androgens). It seems possible from these data that the AR is, in fact, intrinsically less specific than the ER. Evidence for this hypothesis was first demonstrated by Wilson and French (1976), who found that the AR showed about 3 to 4% cross-reaction with estrogens; in 1997, Gaido et al also published data indicating that E2 had approximately 4% the potency of DHT, and 5.5% the potency of testosterone, in their yeast-based assay (Gaido et al, 1997). The reduced specificity of this androgen assay, compared to the estrogen assay, may therefore be a function of the general promiscuity of the AR, and is obviously not restricted to this particular strain of yeast.

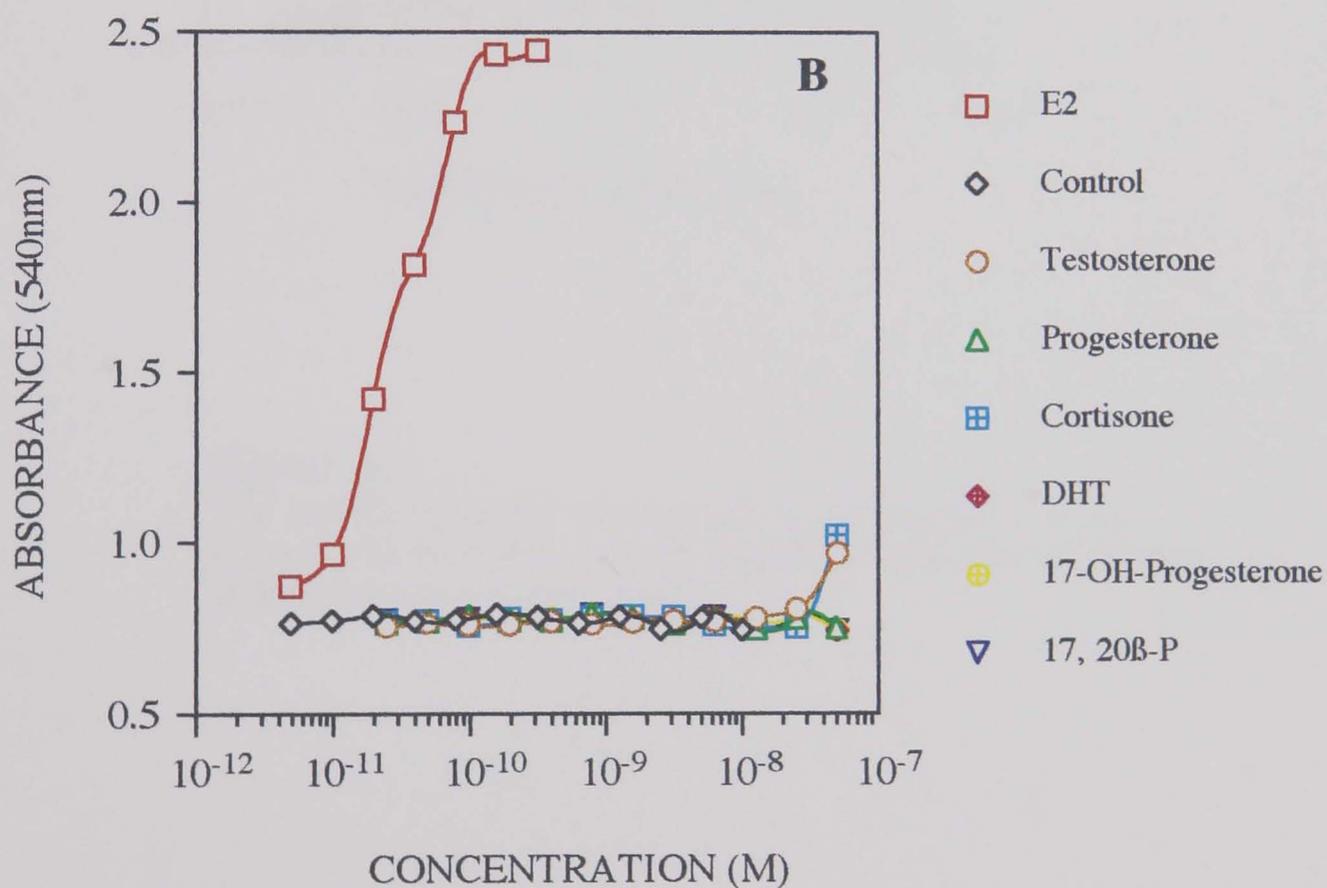
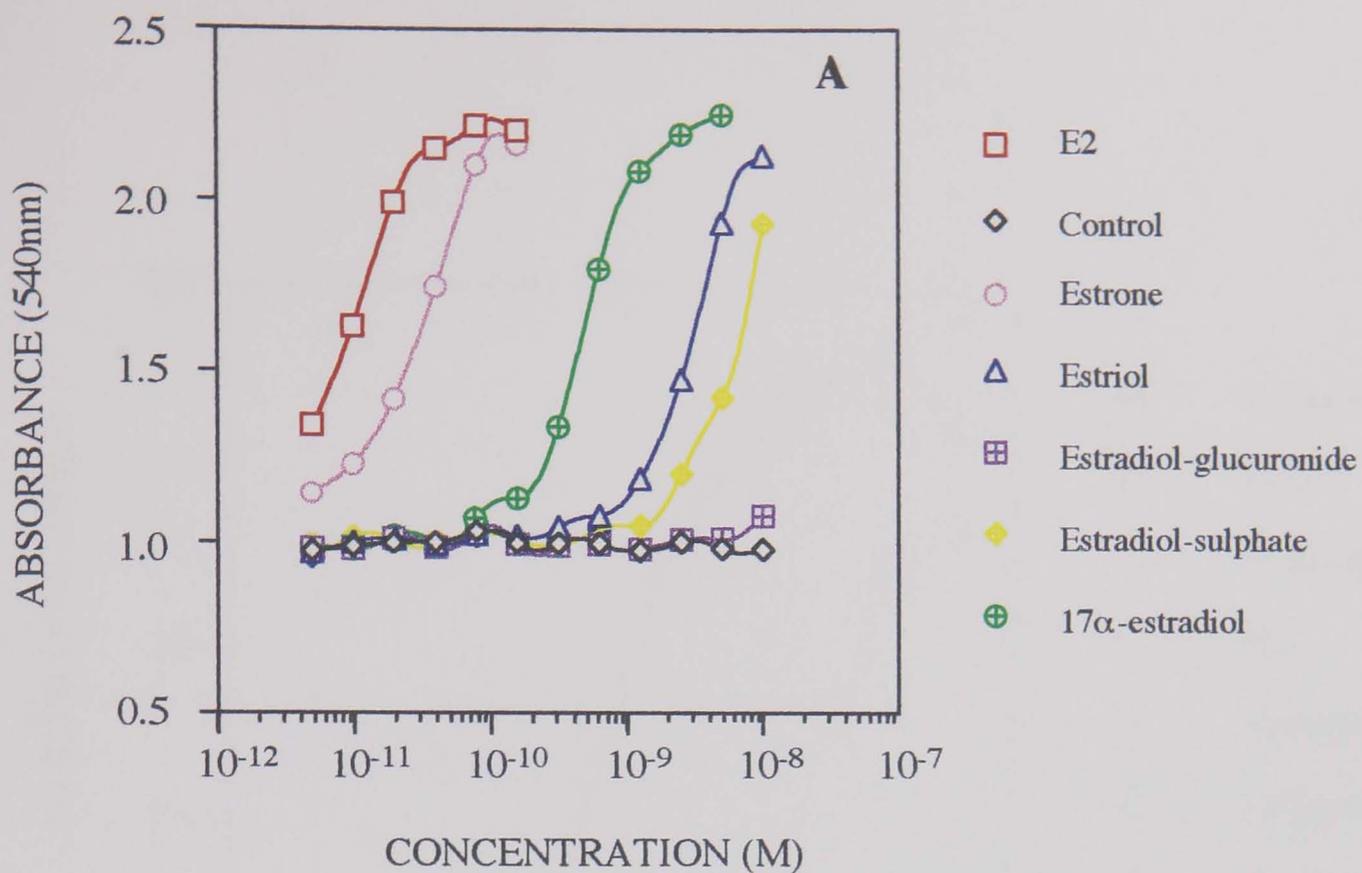


Figure 2.2

Specificity of the recombinant yeast estrogen assay to (A) Estrogens, and (B) Androgens, progesterones and corticosteroids (adapted from Routledge & Sumpter, 1996).

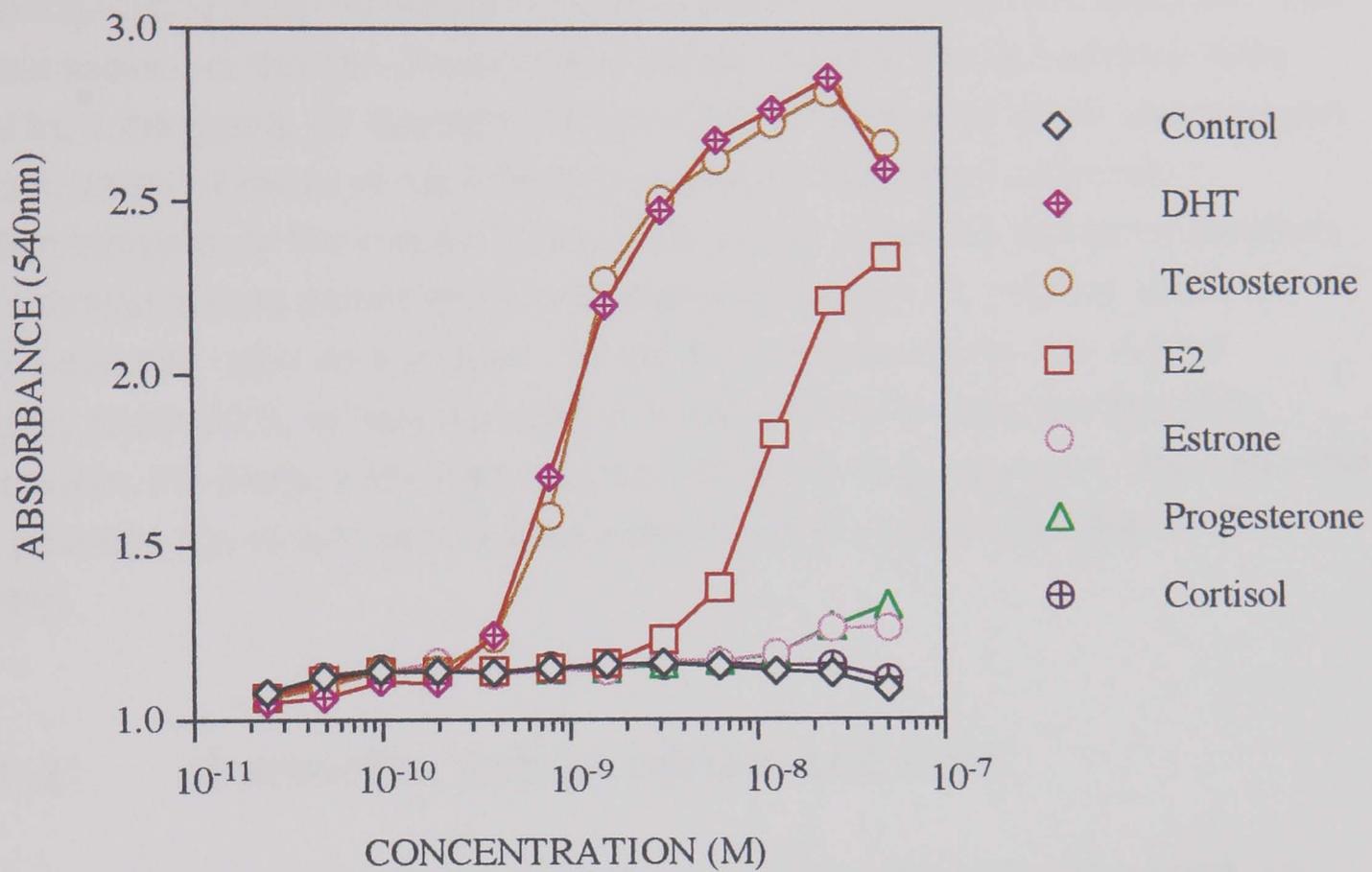


Figure 2.3

The specificity of the recombinant yeast androgen assay. E2 was just over one order of magnitude less potent than the androgens tested in this assay.

2.1.1.4 Calculation of potencies of test chemicals

The potencies of chemicals tested can be compared to that of the relevant steroid by extrapolating from the standard curve at 50% of the maximum response. The concentrations of the test chemical and the standard at this absorbance were read from the graph, as illustrated in figure 2.4. The potency of the chemical can be calculated by either of the following equations: $ED_{50}(\text{test chemical}) / ED_{50}(\text{standard})$; or the inverse of this. The former describes the factor by which the chemical is less potent than the standard in the screen, and the latter expresses this value as a fraction. Where the maximum response did not actually reach 50%, as was the case with many of the estrogenically-active phthalates, the same calculation was carried out at 25% response, and if this was not possible, the response was simply described as greater than that of the control.

2.1.2 Mammalian breast cancer cell lines

Estrogen responsive human breast cancer cell lines were used to confirm any estrogenic activity of the major volume use phthalates that was observed in the yeast screen. It was considered that in the case of the phthalates, cells of human origin may be of particular relevance in assessing activity, since human exposure to these chemicals is potentially extensive. The MCF-7 cell line, in particular, has been demonstrated to be responsive to estrogens by its estrogen dependency to grow as a tumour in athymic mice (Soule and McGrath, 1980; Soto and Sonnenschein, 1985) and was developed as a bioassay to detect estrogenic xenobiotics by Soto et al (1992). This type of cell line has since been reported as a screen which can be used to identify the estrogenic activity of environmental chemicals (e.g. Sonnenschein et al, 1995; Klotz et al 1996).

2.1.2.1 Assay procedure

Cells were cultured in phenol red-free medium containing 5% v/v charcoal dextran stripped serum (DCC). Cells were plated in 6 well plates (Falcon, Becton Dickinson, Lincoln Park, NJ) into this medium 3 to 4 days prior to the experiment. Two experimental designs were used. The first investigated the ability of the chemical to induce proliferation of the cells over time, and involved replacing

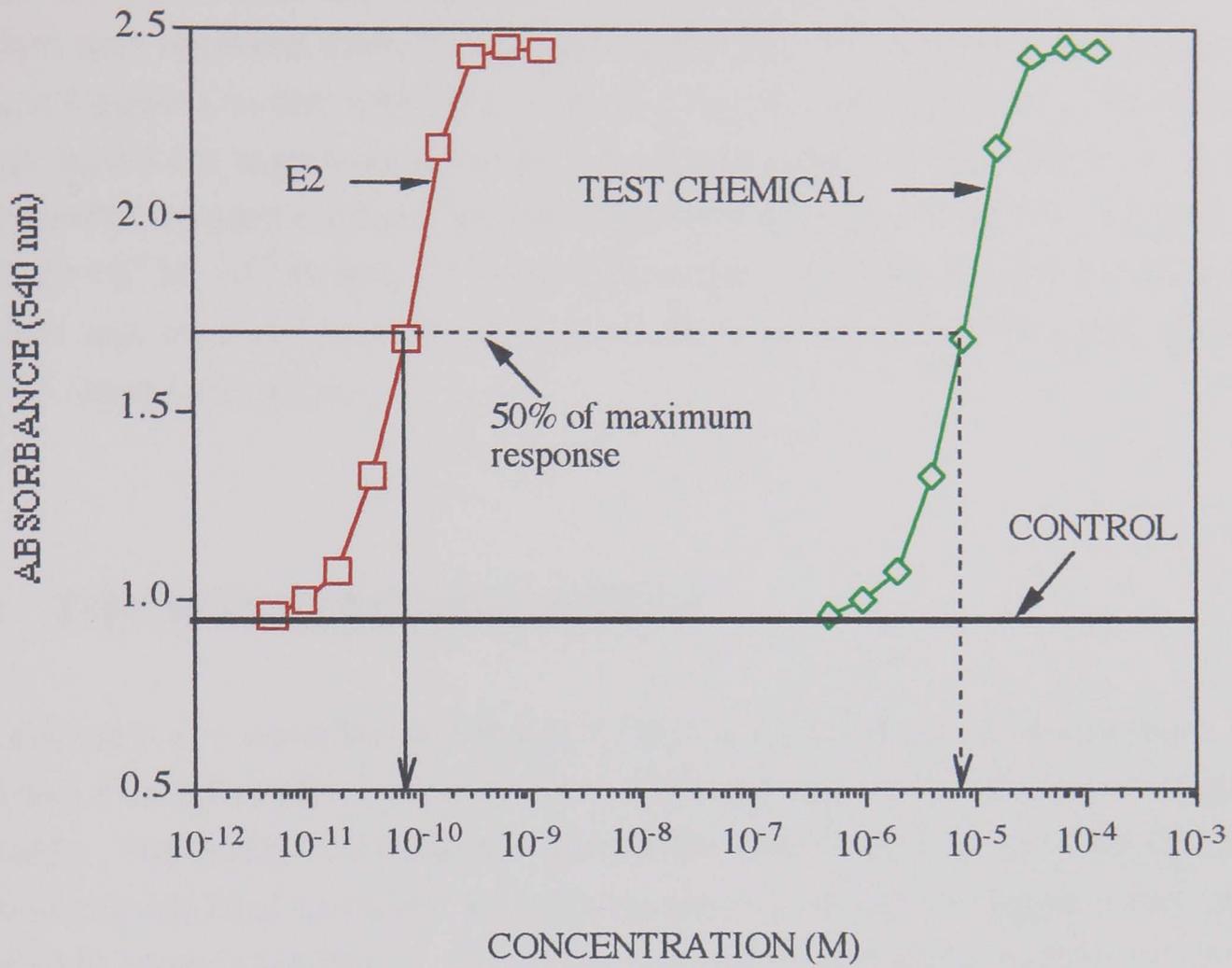


Figure 2.4

An illustration of how the potency of a chemical compared to that of E2 can be calculated. In this case, the ED50 of E2 is approximately 8×10^{-11} M, at an absorbance (at 540 nm) of 1.7. A concentration of 8×10^{-6} M of test chemical is required to produce the same magnitude of response. This chemical is therefore $8 \times 10^{-6} / 8 \times 10^{-11}$ (=100,000) times less potent than E2. Put another way, the chemical induces a response which is 0.00001 times that induced by E2.

medium with treated medium containing either 0.1% vehicle solvent (ethanol), 10^{-8} M E2, or 10^{-5} M of test chemical. Treatments were applied in duplicate. Medium was renewed every 3 days, and cells were trypsinized and counted using a Coulter Counter after 0, 2, 5, 8, and 12 days of incubation. The second design tested the response of the cells to varying doses of test chemical. In these experiments, treated medium contained vehicle solvent, 10^{-8} M, 10^{-10} M, and 10^{-12} M E2, or 10^{-5} M, 10^{-6} M, and 10^{-7} M test chemical. Treatments were in triplicate, medium was renewed every 3 days, and cells were counted at a single endpoint after 11 days incubation.

2.2 THE RADIOIMMUNOASSAY

The principle of a radioimmunoassay is based upon the equal competition between a radiolabelled ligand and an unlabelled ligand for binding to a specific antibody. The amount of radiolabelled ligand and the amount of antibody used in an assay are kept constant, such that when the mixture of reagents has been allowed to reach equilibrium, the amount of radiolabelled ligand which is bound to the antibody will be dependent upon (and inversely proportional to) the amount of unlabelled ligand in the mixture. The maximum amount of label which can bind to the antibody (Maximum Binding, or 'MB') is determined by incubating without any standard or unknown sample. Small amounts of label will also behave as bound ligand, even in the absence of antibody; this is known as non-specific binding (NSB). Reasons for this include factors such as binding of the label to other assay components, trapping of label in the bound complex, presence of impurities in the label, absorption of free tracer to the reaction tube, or the incomplete aspiration of the unbound fraction from the tube. These values, MB and NSB, essentially represent the upper and lower limits of the assay.

2.2.1 The standard curve

With each assay, a standard curve is included in order to determine the concentration range over which the amount of unknown ligand can be quantified.

The assay is set up with fixed amounts of antibody and label, but with serially diluted concentrations of a known amount of unlabelled standard. The NSB value is subtracted from the MB value, and the corrected MB is taken as 100%. The remaining values for the standard tubes are also corrected for NSB and are expressed as a percentage of MB. The data are plotted on a logarithmic scale and a sigmoidal curve is produced. At the lower end of the curve (where high concentrations of standard are present), the competition is strongly in favour of the standard, and hence relatively little label binds. At the upper end, there is relatively little standard, and hence the competition is strongly in favour of the label (and so much of the antibody binds to the label). The central region of the curve is that over which we can extrapolate sample counts to sample concentration. This is where a very small change in concentration of unlabelled ligand produces a large change in amount of label bound to the antibody, and where a straight line can be drawn through the points of the standard curve. An example of a typical standard curve is shown in figure 2.5.

2.2.2 Separation methods

There are two main methods used for the separation of the free unbound label from the antibody-ligand complex at the end of the radioimmunoassay.

The first, used more often in protein assays, is the double antibody method. This involves using a second antibody - raised in a different species than the primary antibody - against gamma globulins from the species used to raise the primary antibody. The second antibody is added to the antibody / unlabelled ligand / label mixture after a period of equilibration, and incubated for at least 16 hours. The second antibody will bind to the antibody-ligand complex and precipitate out of solution, thus halting the dynamic interaction. The precipitate can then be compacted by centrifugation, followed by aspiration of the supernatant. The amount of bound label in the precipitate is inversely proportional to the amount of unlabelled ligand in the sample.

The second separation method involves the use of activated charcoal, and is most often used in steroid assays. Activated charcoal essentially has an increased surface area due to the presence of micropores (<1 nm) and mesopores (1 to 25 nm), therefore small molecules will be adsorbed onto the

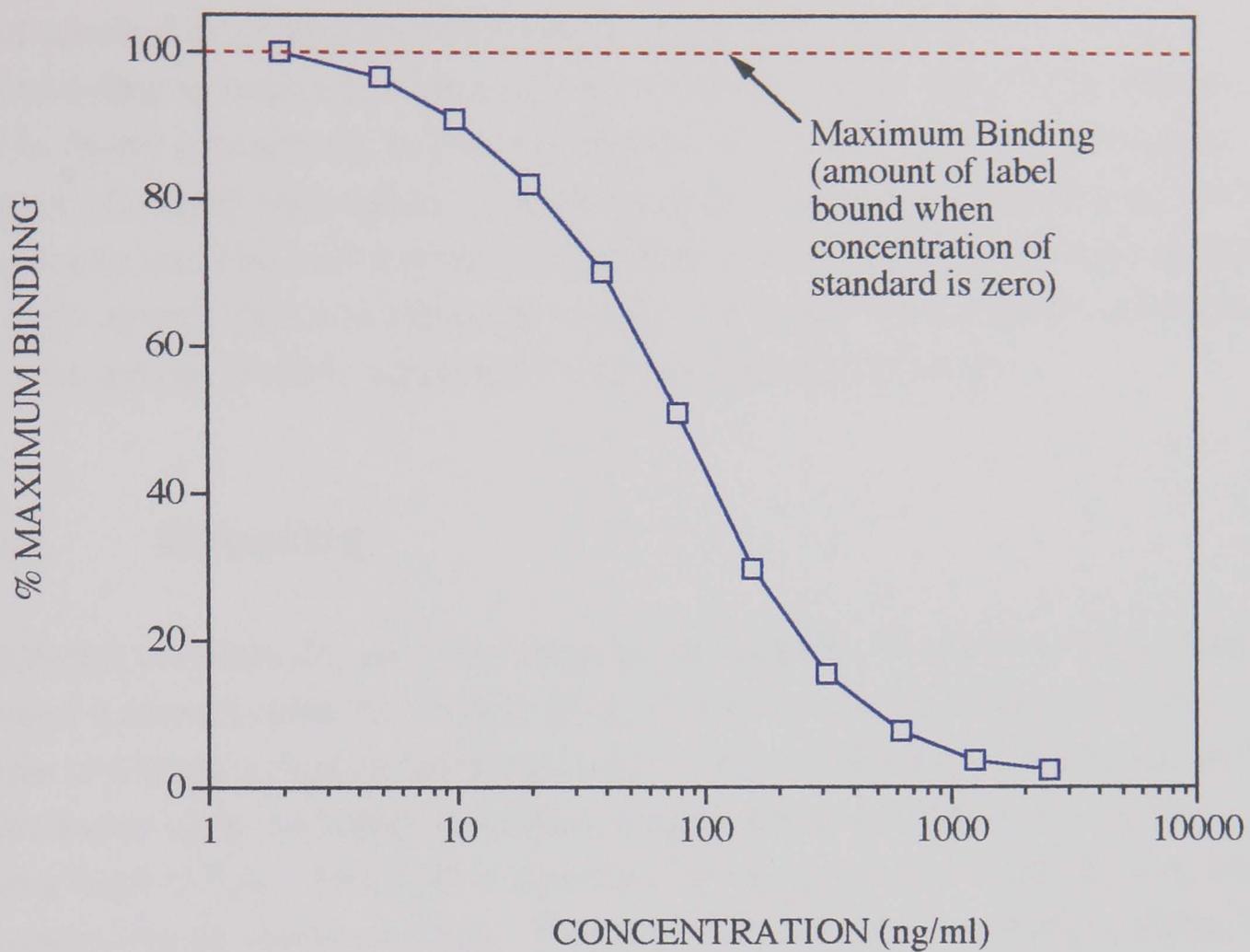


Figure 2.5

An example of a typical standard curve obtained in a radioimmunoassay. The concentration of the required protein / hormone in the 'unknown' sample can be extrapolated from its % Maximum Binding, along the straight region of the curve, which lies approximately between 80% and 20% of Maximum Binding.

charcoal. The unlabelled ligand will thus be adsorbed by the activated charcoal, and this complex will be precipitated out by centrifugation. The antibody (with bound labelled or unlabelled hormone) remains in the supernatant, which is decanted directly into beta vials and retained for counting. Albumin or dextran is used to fill the mesopores, to prevent adsorption of the bound antibody-ligand complex into these larger sites. The timing of the reaction and temperature of the assay tubes must be kept constant to avoid excessive variation between assays. In a large assay, NSB and MB tubes are also included in each batch of samples within the assay, to allow adjustment for batch to batch variation.

2.2.3 Counting

Scintillation counters are used to measure radioactivity. In the case of gamma radiation (generally used for protein RIAs), gamma rays are emitted from the sample and have a high penetrating power; they can therefore travel through the plastic assay tube, and strike a thallium coated sodium iodide crystal, which emits a flash of light. This light is detected by surrounding photomultipliers, and converted into an electrical pulse. The amplitude of this pulse is proportional to the intensity of the scintillant and therefore to the energy of the radiation which produced it. In steroid radioimmunoassays, beta radiation is more often used. This is particulate, and is not highly penetrative, so cannot pass through the plastic of the tube. In this case, photons of light are generated within the vial, by a liquid scintillant which is activated by the radiation. These photons pass through the vials, such that they can be detected by photomultipliers. This method reduces the efficiency of the measurement due to the presence of impurities - such as proteins or coloured chemicals - in the sample, an effect known as quenching.

2.3 PROTEIN RADIOIMMUNOASSAYS

2.3.1 Protein assay buffer (PAB)

280 ml 0.5 M Na_2HPO_4 and 40 ml 0.5 M NaH_2PO_4 was mixed together to form 0.5 M NaPO_4 (pH 7.5), this was added to 2 litres of distilled water. To this solution, 35 g NaCl, 11.7 g EDTA (not the disodium salt), and 4 g NaN_3 was added, and the solution was stirred vigorously for 30 minutes. 20 g egg albumin was dissolved in 600 ml distilled water. This was added to the mixture, together with a further 1 litre of distilled water. The solution was stirred for another 2 hours, and the pH of the mixture was adjusted to pH 7.05 by adding 5 M NaOH slowly, whilst taking continuous readings. The buffer was stirred for a further 1.5 hours and the pH was checked once more. The buffer was filtered, and stored at 4°C.

2.3.2 Purification of label

The proteins used in these assays (vitellogenin (VTG), follicle stimulating hormone (FSH), and luteinising hormone (LH)) were labelled with ^{125}I . Over time, the iodine will detach itself from the protein, and this free radioactivity must be removed prior to the addition of the labelled protein to the assay, to increase sensitivity. A PD10 column, containing Sephadex G25 (Pharmacia), was used to achieve this. An appropriate amount of label was purified each time. The label was eluted with PAB, whereby 2 radioactive peaks were obtained; the first peak was the required labelled ligand, the second was free iodine, as depicted in figure 2.6. The labelled ligand could then be diluted as required.

2.3.3 The vitellogenin radioimmunoassay

2.3.3.1 Vitellogenin standard

Purified rainbow trout vitellogenin was obtained from plasma by gel-filtration chromatography on a Sepharose 6B column (Pharmacia, Milton Keynes, Bucks, UK), prior to ion exchange chromatography on DEAE cellulose (Tyler and

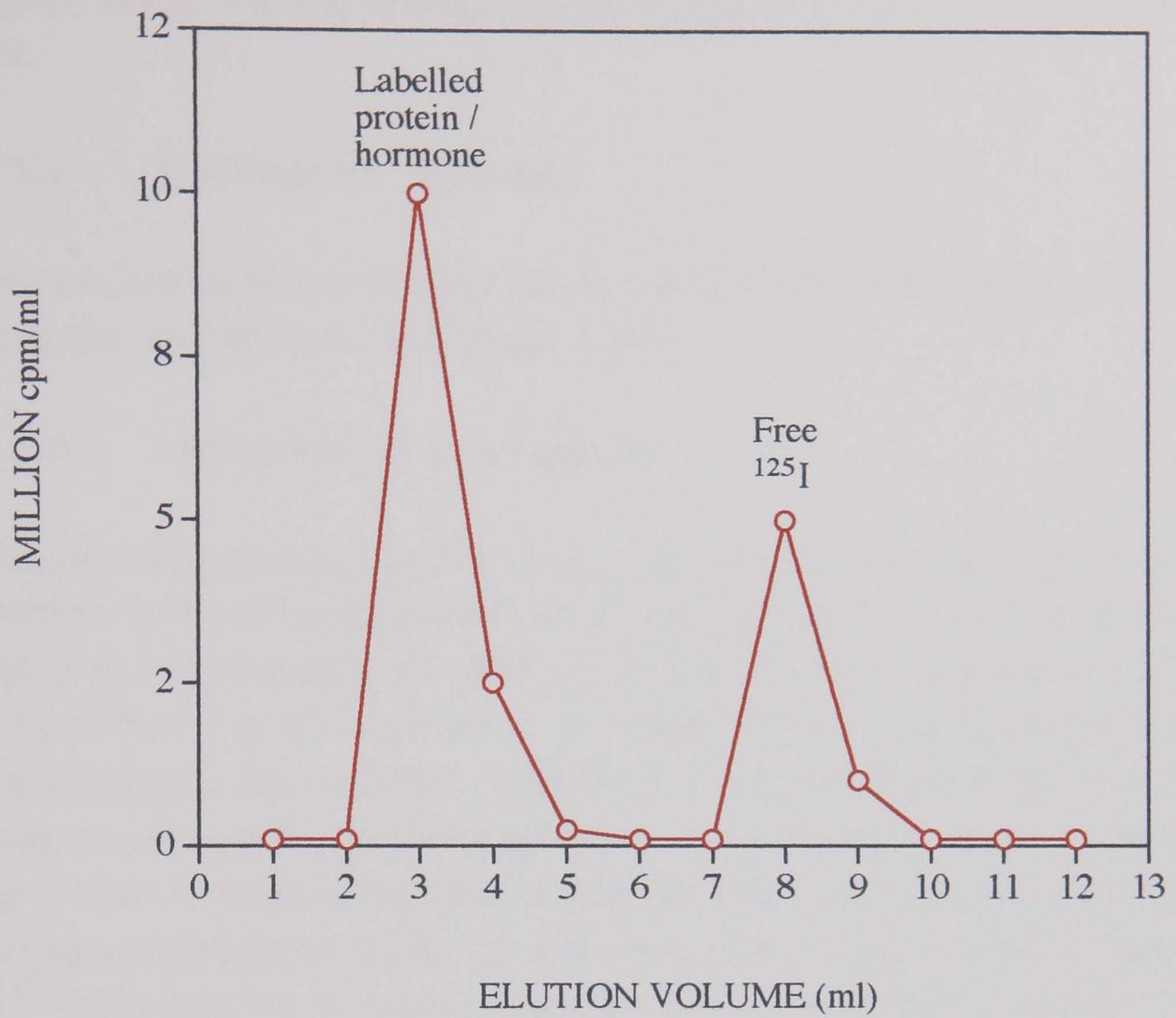


Figure 2.6

Purification of ¹²⁵I labelled protein / hormone prior to use. Label was eluted through a PD10 column, and collected in 1 ml aliquots. Two radioactive peaks were collected, the first being labelled protein, which was retained, and the second was free ¹²⁵I.

Sumpter, 1990). Purified vitellogenin was stored at -20°C in 500 µl aliquots of 10 µg/ml.

2.3.3.2 Vitellogenin antibody

Antibodies were raised in half lop rabbits injected with purified rainbow trout vitellogenin, as described by Sumpter (1985).

2.3.3.3 Iodination of vitellogenin

Vitellogenin was labelled with ^{125}I on tyrosine residues using Iodogen (1,3,4,6-tetrachloro-3 α 6 α -diphenylglycoluril) as an oxidising agent. 5 µg rt-VTG (in 20 µl 0.1 M sodium phosphate) was added to 10 µl 0.5 M sodium phosphate (pH 7.5) and 1 mCi Na ^{125}I on the edge of a 1.5 ml eppendorf vial. The eppendorf vial contained 2 µg Iodogen dried into its base from 20 µl of dichloromethane. The vial was stood upright to allow the mixture to run to the base, and the reaction began. After 10 minutes, the reaction was terminated by the addition of 0.5 ml 0.1 M sodium phosphate buffer (pH 7.5). This mixture was purified to separate free ^{125}I from labelled vitellogenin using the procedure described above (2.4.2), and the labelled vitellogenin was stored at 4°C prior to use.

2.3.3.4 Routine assay plan

Duplicate tubes were set up as follows:

Tubes	1,2	Total Counts
Tubes	3,4	NSB
Tubes	5,6	MB
Tubes	7,8	2500 ng/ml VTG standard
Tubes	9,10	1250 ng/ml
Tubes	11,12	625 ng/ml
Tubes	13,14	312.5 ng/ml
Tubes	15,16	156 ng/ml
Tubes	17,18	78 ng/ml
Tubes	19,20	39 ng/ml
Tubes	21,22	19.5 ng/ml
Tubes	23,24	9.8 ng/ml

Tubes	25,26	4.9 ng/ml
Tubes	27,28	2.4 ng/ml
Tubes	29,30	Unknown
Tubes	31,32	Unknown
...etc		

2.3.3.5 Protocol

A sensitised 4 day assay was used throughout. The assays were performed as follows:

Day 1

Plasma samples were diluted in PAB. 50 µl aliquots of each sample were added to reaction tubes (LP2, Luckhams). 100 µl PAB was added to Total Count tubes and 50 µl to MB, NSB, and all but the first pair of standard tubes. Standards were serially diluted from the second to the eleventh standard, diluting one entire duplicate row before starting the next. 50 µl of rt-VTG antibody (diluted 1:100,000 in 1:400 NRS) was added to each tube except the Total Counts and the NSB tubes. 50 µl 1:400 NRS was added to the NSB tubes. Tubes were vortexed, centrifuged briefly to ensure the contents were in the base of the tube, and incubated at 4°C overnight.

Day 2

Labelled VTG was purified as described above (2.3.2), prior to the addition of 50 µl label containing 20,000 to 25,000 cpm/50 µl to each tube. Tubes were vortexed, centrifuged briefly, and incubated overnight at 4°C.

Day 3

50 µl of second antibody (diluted 1:50 in PAB only) was added to each tube. Tubes were again vortexed and centrifuged briefly, and incubated at 4°C for at least 16 hours.

Day 4

Assay tubes were centrifuged at 4°C for 45 minutes at 3000g. The supernatant was aspirated and discarded from all tubes except the total count tubes. Pellets can be easily disturbed, so care was taken over this procedure, and tubes were kept at 4°C between centrifuging and aspirating if necessary. Each tube

(containing antibody-bound labelled or unlabelled VTG) was counted on a gamma counter for 2 minutes. The detection limit of this sensitised RIA was 11.2 ng/ml (n=9).

2.3.4 The FSH radioimmunoassay

The FSH radioimmunoassay was run on exactly the same principles as the VTG assay, but substituted FSH standard, FSH label, and FSH antibody for the corresponding VTG components, as outlined below.

2.3.4.1 FSH standard

FSH was purified from rainbow trout pituitary glands according to the method described by Swanson et al (1991).

2.3.4.2 FSH antibody

FSH antibody was raised in rabbits against FSH β -subunit isolated from coho salmon (see Prat et al, 1996, for details)

2.3.4.3 Iodination of FSH

Intact FSH was labelled with ^{125}I using the method described above for iodination of VTG (2.4.3.3). A stock solution of 5 μg FSH in 10 μl 0.05 M NaPO_4 (pH 7.5) was used for the reaction.

2.3.4.4 Routine assay plan

The set up of the FSH assay was the same as that for the VTG assay (see 2.4.3.4), except that the standard curve of 11 standards ranged between 100 and 0.1 ng/ml, and therefore the “unknown” tubes started from tube number 29.

2.3.4.5 Protocol

The assay protocol also followed that used for the VTG assay (2.4.3.5), although the activity of the FSH label added to each tube was approximately 3000 cpm.

The assays described in this thesis were undertaken using a standard - and a label made with this standard - which had been freshly purified from rainbow trout (Santos et al, in prep.); the original assays described in Prat et al (1996) used GTH preparations from coho salmon, *Oncorhynchus kisutch*, a species which is closely related to rainbow trout. The resulting assay was slightly more sensitive than that described by Prat et al, and had a detection limit of 1 ng/ml. The specificity of this assay was adequate, but the FSH assay is not as specific as the LH assay, and there was a <10% cross-reaction with homologous hormones (LH, free GTH α , free FSH β ; Santos et al, in prep).

2.3.5 The LH radioimmunoassay

The LH radioimmunoassay was also performed using the same procedure as the VTG assay, except substituting LH standard, LH antibody, and LH label for the respective VTG components, as described below.

2.3.5.1 LH standard

LH purified from coho salmon was used in these assays, as supplied by Dr. Penny Swanson (Swanson et al, 1991).

2.3.5.2 LH antibody

Antibodies against the β -subunit of LH were raised in rabbits as described by Prat et al (1996).

2.3.5.3 Iodination of LH

Iodination of purified LH was performed using the Iodogen method as described above (2.4.3.3), using a stock of 2 μ g LH contained in 10 μ l 0.05 M NaPO $_4$ (pH 7.5).

2.3.5.4 Routine assay plan

LH standard was diluted over 10 steps from 10 ng/ml to 0.02 ng/ml for the standard curve. "Unknown" samples therefore started from tube 27 of the assay.

Otherwise, the assay plan was the same as described for the VTG radioimmunoassay.

2.3.5.5 Protocol

The assay protocol also followed that used for the VTG assay (2.4.3.5), although the activity of the LH label added to each tube was approximately 5000 cpm. This assay was both sensitive and highly specific, with a detection limit of 0.26 ng/ml, and a cross-reactivity (with FSH) of <0.001% (Prat et al, 1996).

2.4 STEROID RADIOIMMUNOASSAYS

The general principal of a steroid RIA has been described in section 2.2.

2.4.1 Steroid assay buffer (SAB)

Buffer was prepared by making 1 litre of 0.05 M phosphate buffered saline (PBS; Oxoid). 1 g of gelatine (Type A: from porcine skin; Sigma) was added to the solution. The mixture was heated on a stirring block until the gelatine had dissolved. The solution was allowed to cool prior to the addition of 1 g sodium azide.

2.4.2 Routine assay plan

Owing to the importance of timing at the separation (using activated charcoal) stage, the addition of charcoal must take place quickly to minimise variability between the first and last tubes; therefore, assays were split into batches, each containing approximately 40 tubes (20 samples). E2 standards were serially diluted from 5 ng/ml to 0.0025 ng/ml, giving 12 dilutions in total, and testosterone standards were diluted from 10 ng/ml to 0.01 ng/ml (11 dilutions). After every 40 tubes, a new "batch" would start, with fresh NSB and MB tubes also. An example of an E2 assay plan would therefore be as follows.

Tubes	1,2	Total Counts
Tubes	3,4	NSB
Tubes	5,6	MB
Tubes	7,8	5 ng/ml E2 standard
Tubes	9,10	2.5 ng/ml
Tubes	11,12	1.25 ng/ml
Tubes	13,14	0.625 ng/ml
Tubes	15,16	0.313 ng/ml
Tubes	17,18	0.156 ng/ml
Tubes	19,20	0.078 ng/ml
Tubes	21,22	0.039 ng/ml
Tubes	23,24	0.02 ng/ml
Tubes	25,26	0.01 ng/ml
Tubes	27,28	0.005 ng/ml
Tubes	29,30	0.0025 ng/ml
Tubes	31,32	Unknown 1
Tubes	33,34	Unknown 2
Tubes	35,36	Unknown 3
Tubes	37,38	Unknown 4
Tubes	39,40	Unknown 5
<hr/>		
Tubes	41,42	NSB
Tubes	42,43	MB
Tubes	44,45	Unknown 6
Tubes	46,47	Unknown 7
...etc		

2.4.3 Assay protocol

Plasma samples were extracted with ethyl acetate prior to assay. This separates the steroids from their sex steroid binding proteins. An appropriate volume of plasma was transferred to a polypropylene tube, followed by 1 ml ethyl acetate. The volume of plasma used here was normally 200 μ l, but dilution rates varied according to the expected plasma E2 or testosterone concentration of the fish at the relevant stage of the reproductive cycle; in addition, a greater volume of plasma was required from the fish exposed to 100 μ g 4-NP/L (see chapter 4) due

to the suppressed steroid titres. The tube was capped, shaken, and stored at -20°C.

Day 1

The samples (plasma plus ethyl acetate) were spun at 2500g for 2 minutes to separate the ethyl acetate (top layer) from the plasma (lower layer). The ethyl acetate, containing the extracted steroid hormone, was decanted into another tube. The ethyl acetate was then removed using a Gyrovap, and the steroid remaining was redissolved in 100 µl SAB. 200 µl SAB was added to the total counts and NSB tubes; 100 µl was added to each standard tube except the first pair. Standard was serially diluted from the second to the last standard, for an entire row, before serially diluting a duplicate row of standards.

100 µl of labelled (tritiated) hormone (approximately 9000 disintegrations per minute (dpm) for E2; approximately 6000 dpm for testosterone) was added to each tube. 100 µl of antibody (diluted 1:200,000 for E2, 1:50,000 for testosterone) was added to all tubes except the total counts and NSB tubes. Tubes were vortexed and centrifuged briefly to ensure the contents were in the base.

Day 2

As mentioned previously (2.2.2), activated charcoal was used to separate the bound complex from the unbound label. Dextran-coated charcoal was prepared (0.5% activated charcoal; 0.05% dextran in SAB) 15 minutes prior to use. 500 µl SAB was added to the total count tubes. 500 µl activated charcoal mixture was added to each of the remaining tubes (one batch at a time), the tubes were vortexed and incubated on ice for 10 minutes prior to centrifuging at 2500g for 15 minutes, at 4°C. The supernatant (containing the bound fraction) was removed and transferred to scintillation vials to which 4 ml scintillation fluid (Liquiscint, National Diagnostics) was added. The tubes were shaken and counted in a β-counter for 5-10 minutes.

Calculations were carried out by hand using the mean dpm for each pair of tubes and applying the following equation: $((\text{unknown-NSB}) / (\text{MB-NSB})) \times 100$. The standard curve was plotted on log-normal paper, and the unknown values were read from this curve.

The 17 β -estradiol assay was both highly specific and highly sensitive; the detection limit was 0.028 ng/ml (n=11); the cross reactivity with other estrogens (estrone and estriol) was <0.5%, and with other steroids was <0.005% (Carragher, 1988). The testosterone assay was similarly sensitive and specific; the detection limit was 0.033 ng/ml (n=3) and the cross-reactivity with other steroids ranged from 2.4% (11-ketotestosterone) to <0.01 (various progesterones) (Scott et al, 1984).

2.5 EXTRACTION AND ANALYSIS OF WATER SAMPLES

Water samples from in vivo experiments (in which fish were exposed to chemicals) were collected at intervals, extracted, and analysed using both analytical chemistry and a bioassay (the recombinant yeast screen). Analytical chemistry provided a more accurate determination of actual concentration of test chemical in the sample, whereas the yeast screen measured the estrogenicity of the water in the dosed tanks compared to that of the control tanks. General techniques followed those described by Janbakhsh (1996).

2.5.1 Collection and storage

Water samples were collected in solvent-rinsed glass bottles and preserved with 10 ml of 38% formaldehyde per litre. Samples were stored in the dark at 4°C prior to extraction, for a maximum of 1 week.

2.5.2 Extraction

Samples were concentrated using solid phase extraction on Sep-Pak cartridges which contain a sorbent packing material to which organic matter in the water samples partitions. In these experiments, Sep-Pak cartridges containing a packing material of 200 mg ethyl bonded silica (C2; JT Bakers, Berkshire, UK) were used. These were reported to have a better ability to recover alkylphenols from water, compared to those containing the frequently used octadecyl (C18) packing material (Janbakhsh, 1996).

A simplified diagram of the procedure is shown in figure 2.7. A 12-port Visiprep solid phase extraction manifold (Supelco, Dorset, UK) was used to extract multiple samples. The cartridges were first primed with 5 ml methanol, followed by 5 ml ddH₂O. The sample was then loaded onto the cartridge and drawn through at a rate of approximately 5 ml/min. The cartridge was washed with 3 ml ddH₂O, and dried by drawing air through the cartridges for 30 minutes. The samples were then eluted with 5 ml methanol. The methanol was evaporated using a stream of oxygen-free nitrogen, and the sample redissolved in an appropriate volume of solvent, depending on the concentration of the original sample and the detection limit of the assay used in the final analysis.

2.5.3 Analysis of extracted samples

Water extracts were analysed for estrogenic activity using the recombinant yeast assay (see section 2.1.1) and for accurate determination of chemical concentrations using analytical chemistry techniques.

Water samples collected from the dosing experiment in which rainbow trout were exposed to varying doses of 4-NP were analysed by normal-phase HPLC, followed by fluorescence detection. The method used is described in Janbakhsh (1996). Briefly, the HPLC system Crystal 200 Liquid Chromatograph (ATI Cambridge) consisted of quaternary solvent proportioning valves and dual piston pump head. The sample injection was carried out by a LC230 auto-sampler (ATI Cambridge) with a 20 µl sampling loop. The LC-NH2 normal phase analytical column (Supelcosil, 25.0 cm, 4.6 mm, 5 µm) was used for separation. The determinands were detected by a FL2000 Spectra-System Fluorescence detector, at excitation and emission wave-lengths of 230 nm and 302 nm, respectively. The data were integrated and recorded by a Unicam 4815 computing integrator (ATI Cambridge).

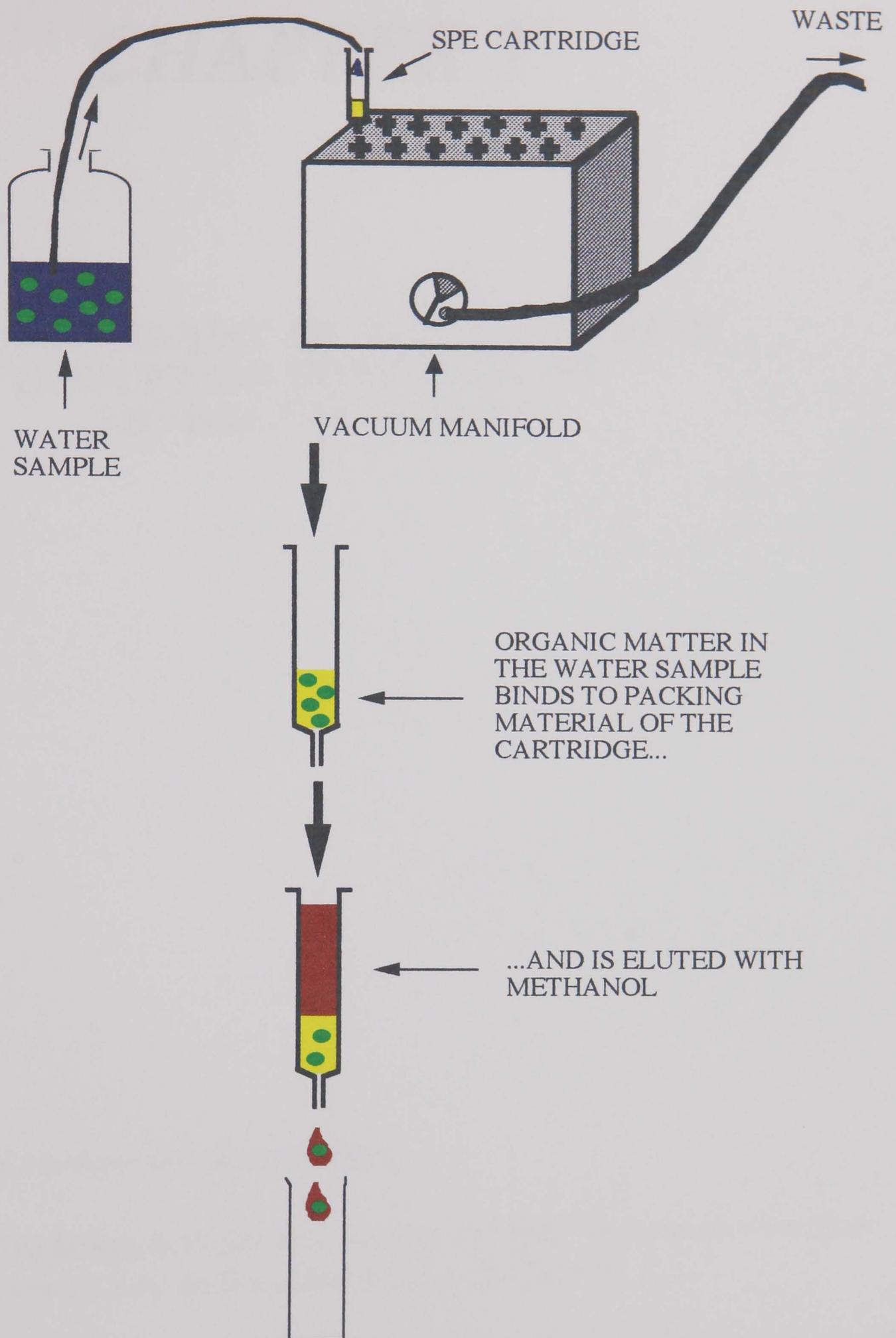


Figure 2.7

An illustration of the extraction procedure employed to concentrate the test chemical present in water samples prior to analysis by HPLC or bioassay.

CHAPTER 3

AN ASSESSMENT OF THE ENDOCRINE DISRUPTING POTENTIAL OF PHTHALATE ESTERS

Data from this chapter have been published in:

Harris CA, Henttu P, Parker MG, Sumpter JP (1997) The estrogenic activity of phthalate esters in vitro. *Environ. Health Perspect.* 105: 802-811

Harries JE, Runnalls T, Hill E, Harris CA, Maddix S, Sumpter JP, Tyler CR (2000) Development of a reproductive performance test for endocrine disrupting chemicals using pair-breeding fathead minnows (*Pimephales promelas*). *Environ. Sci. Technol.* 34: 3003-3011

3.1 INTRODUCTION

3.1.1 The abundance of phthalates in the modern environment

Phthalate esters are among the most widely used industrial chemicals in existence. They are used principally as plasticisers, to impart flexibility, workability, and durability to polymers, but they can also be found in products such as paints, adhesives, inks and cosmetics. Millions of tonnes of phthalates are produced world-wide every year, with hundreds of thousands of tonnes being used in Europe alone (see table 3.1). The significance of phthalate consumption lies in the fact that their applications are widespread and extremely diverse. This, together with the fact that their very nature determines that they are fluid within the materials to which they are added, and thus can leach from these media, leads to the ubiquitous presence of phthalates in environmental samples. On the other hand, phthalates are far more reactive than many other industrial organic contaminants with low solubilities, and therefore degrade more easily in the environment; thus although the input is constant, the final concentration may be lower than initially expected. Consequently, major questions that arise are "what do they degrade to?", and "are the intermediate degradation products harmful in themselves?"

Name	Abbr.	Molecular Weight	Solubility (mg/L) *	log K _{ow} *	Mass Consumed in Europe (tonnes/annum)
Di(2-ethylhexyl) phthalate	DEHP	390.56	0.003	7.5	400-500,000
Diisononyl phthalate	DINP	425	<0.001	>8.0	100-200,000
Diisodecyl phthalate	DIDP	447	<0.001	>8.0	100-200,000
Butyl benzyl phthalate	BBP	312.39	2.7	4.59	20-50,000
Dibutyl phthalate	DBP	278.35	11.2	4.45	20-40,000
Diisobutyl phthalate	DIBP	278.35	20.0	4.11	20-40,000

Table 3.1

Details of the high volume use phthalates (>20,000 tonnes/annum in Europe). An asterisk indicates that the data were taken from Staples et al (1997).

The basic structure of a phthalate ester comprises an aromatic ring with 2 aliphatic side chains. There are more than 60 different phthalates in use. Generally, however, only a small number are used in large quantities in Europe; these are listed in table 3.1, and their structures are shown in figure 3.1. As a class, the popularity of phthalates as plasticisers is owed to their inertness, fluidity, and high solubility in the polymer (Giam et al, 1984). Also significant are their low water solubilities, and low volatilities, which help prevent loss from the polymer and thus ensure the continued flexibility of the plastic. The lengths of the side chains determine the properties of each individual phthalate, and therefore its end use.

Di-2-ethylhexyl phthalate (DEHP) is by far the most widely employed phthalate, and hence much of the research into the behaviour and toxicity of this class of chemicals has focused on DEHP and its metabolites. The major end use of DEHP is as a plasticiser in polyvinyl chloride (PVC), in which it can frequently be found forming up to 40% of the end product. Applications of this type of PVC include such human contact materials as childrens' toys and blood bags. Other phthalates used in large volumes that will also feature prominently in this chapter include butylbenzyl phthalate (BBP), which is primarily used to make vinyl floor tiles, but has alternative applications, for example in car upholstery and adhesives, and dibutyl phthalate (DBP), used primarily as a plasticiser, but which also has a wide variety of non-plasticiser applications, such as an ingredient in paints, inks, glue, nail polish, hair spray, and insect repellents (Lyons, 1995). Diisononyl phthalate (DINP) is also currently under investigation, not only because it is a high volume use phthalate, but also because a number of recent studies have discovered significant concentrations (constituting up to 50% of the product) in childrens' toys, many of which are designed specifically for sucking by young children; for example, teethers (Stringer et al, 1997; Rastogi, 1998). Since very young children are known to be at a very sensitive stage of development, it is important to know that they are safe from any chemicals to which they may be exposed, be it in low doses chronically, or in a high acute dose. There are currently many calls from consumer groups to ban the use of phthalates in toys, and several retailers already have a voluntary commitment to removing such products from their shelves. Hence, the use pattern of phthalates as discussed here may well alter in the near future.

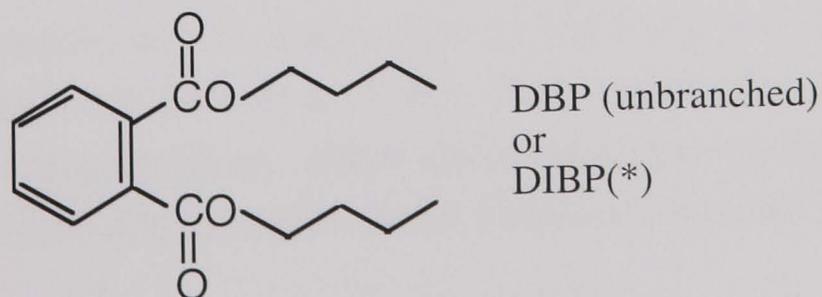
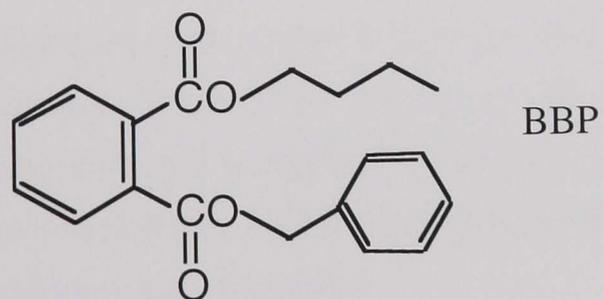
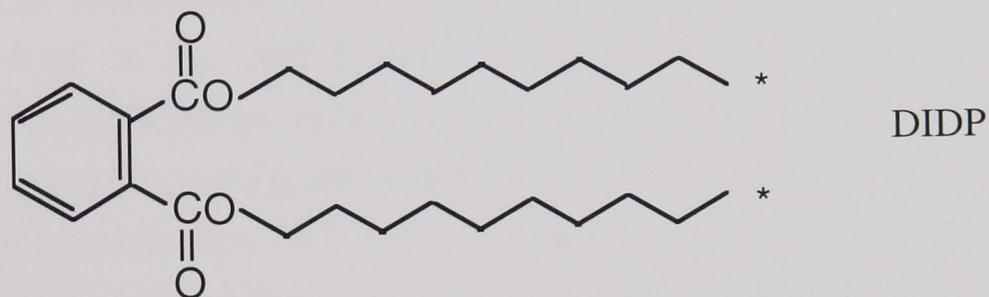
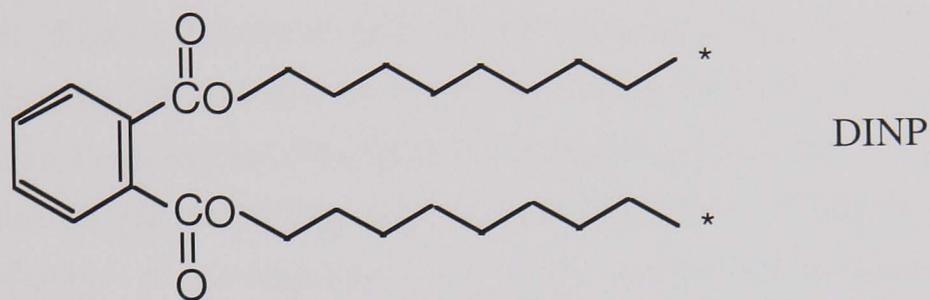
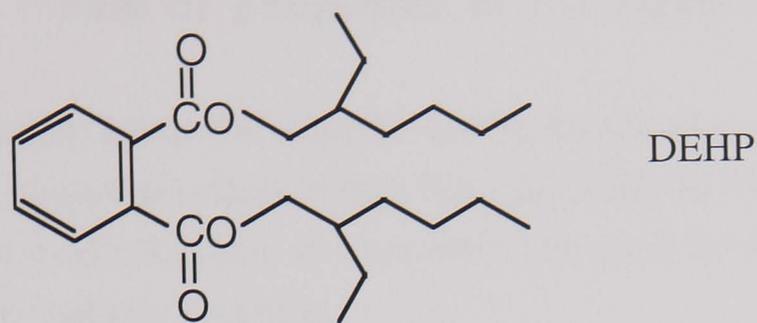


Figure 3.1

The structures of the high volume use phthalates. An asterik indicates that these phthalates may consist of various isomeric forms, due to branching of the alkyl side chain.

3.1.1.1 Fate of phthalates in the aquatic environment

Phthalates can enter the aquatic environment via a number of pathways. These include in industrial waste direct from processing plants, in leachate from disposal of end products, in domestic and industrial sewage effluents, and in waste from vehicle washing.

It is generally accepted that biodegradation of phthalates in water is relatively fast (the half life obtained from shaking flask studies being reported as a matter of days, as opposed to months or years for some chemicals (Sugatt et al, 1984)), and therefore measured concentrations of the parent compounds are, on the whole, in the range of the low $\mu\text{g/L}$ to undetectable. However, this does not rule out accumulation of phthalates in the often anaerobic sediments of aquatic environments, which may lead to exposure of sediment dwelling or feeding organisms. Indeed, the log K_{OW} values (see table 3.1 for some examples) calculated for phthalates (ranging from 7.5 for the longer chain DEHP, to 4.45 for DBP, and 1.61 for the short chain dimethyl phthalate (DMP) (Staples et al, 1997) suggests that phthalates, particularly those with longer side chains, may have a tendency to bind to particulate matter, which will settle out to form sediment. This is supported by the data of Long et al (1998), who detected concentrations of DEHP in water around the low $\mu\text{g/L}$ mark, with the highest measurement being 21 $\mu\text{g/L}$, whereas in the same rivers, concentrations of up to 115 mg/kg were measured in suspended sediment samples, with a general trend of higher concentrations of phthalates in suspended sediments than in bed sediments being observed. The concentration of DEHP in the sediments of all but two samples was in the low mg/kg to the 10's of mg/kg range, therefore being at least 3 orders of magnitude higher than the concentration measured in the corresponding water samples.

Sewage effluent samples have also been found to be contaminated with phthalate residues. Concentrations of DEHP up to 245 $\mu\text{g/L}$ were found in Scottish effluents (Pirie et al, 1996). In that particular study, DEHP was detected in 59% of samples taken. Other phthalates, such as DEP, DBP, DIBP, and BBP, were also detected, but at reduced frequency and far lower concentrations.

3.1.1.2 Fate of phthalates in the terrestrial environment

Phthalates have also been reported in samples taken from various compartments of the terrestrial environment, including the atmosphere, sludges, soils, landfill sites, and plants.

One route of entry of phthalates into soil systems is via the application of sewage sludge to soil. Large volumes of sludge are disposed of to agricultural land, providing beneficial soil fertilising and conditioning properties. Another potential route of entry is via fallout from the atmosphere, as discussed by Thuren and Larsson (1990). They reported mean air concentrations of DEHP and DBP in the low ng/m³ range, similar to those detected by Giam et al (1980) in the atmosphere of the Gulf of Mexico. The latter study found DEHP and DBP to be associated with both the particulate and the vapour phase in the air.

A summary of the properties of individual phthalates controlling their fate in the environment (such as solubility, log K_{OW}, and vapour pressure) can be found in Staples et al (1997); solubilities and log K_{OW} values of the high-volume use phthalates can be seen in table 3.1.

3.1.1.3 Degradation of phthalates in the environment

Despite the constant input and widespread distribution of phthalates into environmental systems, the rapid degradation of these chemicals is of paramount importance in preventing, in most instances, their accumulation. As a rule, biodegradation pathways are dominant in surface waters, soils, and sediments, whereas photodegradation prevails in the atmospheric compartment (Staples et al, 1997).

The degradation rates of phthalates are dependent on their molecular weight; those with longer alkyl side-chains tend to have longer half-lives in a given environment. This phenomenon was observed by Shelton et al (1984), who found DnOP and DEHP to remain intact in anaerobic digester sludge, whereas the lower molecular weight DMP, DEP, and DBP were completely mineralised, as was BBP, albeit at a slightly reduced rate. A similar picture was reported by Ejlertsson et al (1996, 1997), who found that more soluble phthalates were degraded in anaerobic conditions, whilst less soluble esters were not. In aerobic

environments, e.g. activated sludge systems (O'Grady et al, 1985), or the modified Sturm test (Scholz et al, 1997), the higher molecular weight phthalates can also be transformed, albeit at a slower rate than those with shorter alkyl side-chains.

It can be concluded that there is a general trend for decreasing biodegradability of phthalates corresponding to increasing molecular weight. Hence, DEHP, the most prolific phthalate in use today, is also one of the most persistent in the environment (Rogers, 1996).

3.1.1.4 Exposure of humans to phthalates

The majority of studies discussing exposure of humans to phthalates involve oral exposure, for instance through ingestion of food, or the chewing of toys by small children. Other potential routes may include skin contact, for example from constantly handling plastic articles, contact with PVC clothing or vinyl childrens' pants, and cosmetics; or inhalation, for example as a result of sitting in a car where phthalates are volatilising from upholstery into the atmosphere of the car interior. However, due to the difficulties associated with quantifying such exposure, there are no actual data on the degree of exposure via these latter two pathways; therefore, whilst they must be borne in mind, the attention here will focus upon oral exposure of humans to phthalates. It must also be considered that any exposure estimates are just that, estimates, and too much weight should not be placed on such subjective analyses.

DINP and DEHP can in many cases make up 10 to 40% by weight of PVC toys, including teethingers which are intended to be put into the mouths of small children (Stringer et al, 1997; Rastogi, 1998). These studies established concentrations of phthalates in the toys, but not their leaching potential. This latter question has been addressed by Steiner et al (1998) and Vinklesoe et al (1997); the former found DEHP leaching from PVC child articles into a saliva simulant, and the latter reported the leaching of a range of phthalates from PVC teethingers. These data demonstrate a real possibility of exposure of young children to phthalates from PVC toys.

Exposure of humans to phthalates via food is also a potential risk, as is evidenced by the number of studies which have analysed phthalates in food

packaging materials (MAFF, 1995; van Lierop, 1997; Nerin et al, 1998), the migration of phthalates from such packaging (Harrison, 1998), and the subsequent concentrations of various phthalates in food products (MAFF, 1996a; MAFF, 1996b; MAFF, 1998; Sharman et al, 1994; Petersen, 1991; Page and Lacroix, 1995). It should be made clear, however, that some researchers consider increased phthalate concentrations to arise not only from packing materials, but also from general environmental contamination during, for example, the processing of the food products (MAFF, 1996a; Sharman et al, 1994). Young children are at a particularly sensitive stage of development, and surveys have been carried out to assess the extent of phthalate contamination in infant formulae. An initial survey found total phthalate concentrations of 1.2 to 10.2 mg/kg (MAFF, 1996b), but a follow-up survey has reported reassuringly lower concentrations in similar samples, of <0.1 to 0.6 mg/kg (MAFF, 1998), thereby lowering the overall exposure of infants fed on these diets to within set safety limits.

The exposure of humans to phthalates is unquantifiable, owing to the ubiquity of these chemicals and the considerable number of potential pathways of exposure, many of which scientists have not even attempted to assess. Therefore, it is not possible to state that the apparently low levels of exposure from food products, however encouraging they are, indicate an intake which is safe overall.

3.1.1.5 Exposure of aquatic organisms to phthalates

There is a distinct lack of research specifically devoted to exposure of aquatic organisms to phthalates. Briefly, however, pathways may include: intake via the gills of fish, uptake through food sources (which may themselves have absorbed phthalates from the water, or ingested food which had done so), intake of suspended sediments, and uptake due to living or feeding in the sediments of the water body floor. Uptake via the gills of fish is potentially an especially direct source of contaminant to the bloodstream, because the blood reaches many organs before passing through the liver, where most metabolism will occur, and thus this route of exposure should be researched more fully.

3.1.1.6 Metabolism and bioaccumulation

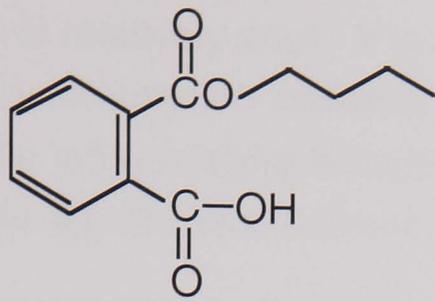
Metabolism of environmental contaminants can have major implications when considering their mechanisms of toxicology. In vitro assays seldom, possibly never, display the same metabolic pathways as whole organisms, and hence, if any effects of toxicants (such as phthalates) are due to metabolites, rather than the parent compounds, then only in vivo studies can provide the necessary information; results of in vitro assays, by themselves, may be misleading.

The primary metabolites of phthalates are their monoesters. The first step in phthalate degradation is hydrolysis, whereby one of the side chains is cleaved, leaving the monoester plus an alcohol group. Examples are shown in figure 3.2.

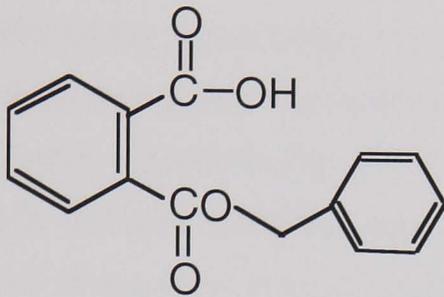
3.1.1.7 Metabolism and bioaccumulation of phthalates in aquatic organisms

Stalling et al (1973) exposed channel catfish to DEHP and found MEHP to be the major metabolite, with 66% of DEHP converted to MEHP after a 24 hour exposure to 1 µg DEHP/L. They also exposed fathead minnows to DEHP, and found that the ratio of MEHP:DEHP was lower compared to that observed in the channel catfish. However, the concentrations of DEHP and exposure periods were different, so the experiments are not comparable (Stalling et al, 1973). In the same study, the authors used hepatic microsomes to examine metabolism of DBP and DEHP. Degradation of DBP reached 97% after 2 hours, whereas only 6% of DEHP was metabolised in the same length of time.

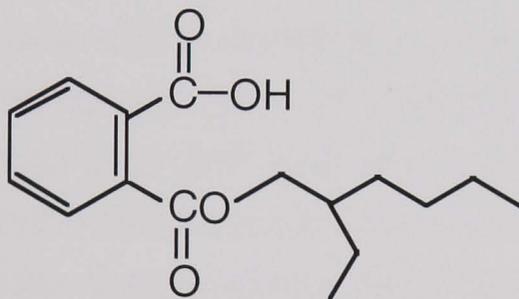
In the Bluegill Sunfish, metabolism of BBP was studied (Carr et al, 1997). The bioconcentration factor (BCF) for intact BBP was found to be 9.4 (for the whole fish). This was compared to estimated BCF values calculated by 4 different groups of scientists, using physical properties such as K_{ow} and solubility, of 3174, 2528, 705, and 304 (cited by Carr et al, 1997), which appear to be vast overestimates. The degradation products of BBP were not characterised by Carr et al (1997). Algae (e.g. *Chlorella pyrenoidosa*) have perhaps higher potential for accumulation of phthalates, but despite their BCFs reaching maxima of 162 (after 24 hours), 205 (after 12 hours), and 4077 (after 12 hours) for DMP, DEP and DBP, respectively (Yan et al, 1995), they were also found capable of metabolising these chemicals to a certain extent.



MBP
(Monobutyl
phthalate)



MBzP
(Monobenzyl
phthalate)



MEHP
(Mono-2-ethylhexyl
phthalate)

Figure 3.2
Structures of the primary metabolites of some of the phthalates.

Despite these studies, our knowledge of metabolism of phthalates in aquatic organisms is relatively poor. It is dangerous to generalise from the limited, and fragmented, information available to all aquatic organisms, both plants and animals, the latter ranging from invertebrates to higher vertebrates, which inhabit the variable aquatic environment.

3.1.2 The reported endocrine activity of phthalates

Certain phthalates have been recognised for many years as reproductive toxicants (a summary of the testicular toxicity of phthalates can be found in Gangolli, 1982). Essentially, the studies report decreased testicular weight, and seminiferous tubular atrophy, induced by DEHP, DBP, di-n-propyl phthalate (DnPP), and di-n-hexyl phthalate (DnHP), as well as by their respective monoesters. With respect to DEHP in particular, further studies by Gray and Gangolli (1986) led them to conclude that MEHP is, in fact, the active testicular toxin produced as a result of metabolism of DEHP in vivo. Ema and colleagues have also undertaken numerous studies in an effort to elucidate the reproductive and developmental toxicity of phthalates to rats exposed in utero. These studies have revealed that BBP and DBP can induce decreased uterine and ovarian weights, and increase postimplantation embryonic loss (Ema et al, 1994, 1997). More recently, the same scientists have found that DBP, administered to rats during the latter half of pregnancy, had an overall adverse effect on the reproductive development of male fetuses (Ema et al, 1998); these data are supported by those of Imajima et al (1997), who demonstrated that rats exposed in utero to MBP (the major metabolite of DBP) exhibited increased incidences of cryptorchidism. Furthermore, a multigenerational study by Wine et al (1997) highlighted effects of DBP on the reproductive development of F₁ generation rats, whereas fewer adverse effects had been seen in the F₀ generation. This latter study was originally published as an internal report for the National Toxicology Program in the US (Gulati et al, 1991). However, following the recent research on the endocrine activities of phthalates, a reassessment of such work has been undertaken, as a possible explanation for the reproductive toxicity of the phthalates has been provided (see below).

3.1.2.1 Estrogenic activity in vitro

The first report of estrogenic activity of phthalates was published as recently as 1995. Jobling et al (1995) assayed a number of xenobiotics in several in vitro assays, and found BBP, DBP, and DEHP competed with E2 for binding to the rainbow trout ER, albeit weakly so. That is, very high concentrations were required, and even at the highest concentration tested, the response was far less than the maximal response elicited by E2 itself. The same scientists investigated these phthalates for their ability to induce proliferation of ZR-75 breast cancer cells - a response specifically stimulated by estrogens. In that assay, only BBP and DBP were found to be estrogenically active, and again, their responses were less than maximal. The third assay performed by Jobling et al (1995) assessed the abilities of the chemicals to stimulate transcriptional activity of the human estrogen receptor. In this assay, as in the former two, BBP was found to be the more potently estrogenic phthalate, DBP was also active, as was DEHP (albeit only at concentrations exceeding 1×10^{-4} M). This pattern of estrogenic activity of these phthalates has been repeated in the majority of studies reporting the results of in vitro assays published since the paper of Jobling et al (1995). There are some exceptions, such as data from Soto et al (1995), who concluded that of all the phthalates tested in the "E-Screen" (monitoring the proliferation of MCF-7 breast cancer cells), only BBP was estrogenic. These authors concluded that the alkyl phthalates were not estrogenic, although it was not clear at what concentrations they had been tested, and therefore it may be that the concentrations of alkyl phthalates used were simply not high enough to stimulate a response. Another example of data contrasting with those reported by Jobling et al (1995) was published by Gaido et al (1997), who found BBP, at concentrations up to 1×10^{-4} M, to be inactive in a yeast-based estrogen assay.

Petit et al (1997) used a novel in vitro yeast-based assay, in which the yeast had been transformed with a gene coding for the rainbow trout estrogen receptor. DEHP was not estrogenic in this assay, but DBP was weakly active. Both of these phthalates were inactive in a competitive binding assay based on the rainbow trout estrogen receptor, and DBP was found not to induce vitellogenin mRNA production (an estrogen-specific response) in hepatocyte cell cultures (Petit et al, 1997). Another recombinant yeast-based assay, this time with the yeast transformed with a gene for the human ER, found BBP to be estrogenically active, but DBP and DEHP were inactive (Coldham et al, 1997). In this study,

BBP was also tested in an in vivo assay, namely the mouse uterotrophic assay (in which the endpoint is relative uterine weight, an increase in uterus weight indicating an estrogenic stimulus) and found to be inactive.

Zacharewski et al (1998) recently reported the results of testing 8 phthalates (DEHP, DBP, BBP, DHP, DIHP, DnOP, DINP, and DIDP) in a battery of in vitro and in vivo assays, and found DBP, BBP, and DHP to be weakly estrogenic in both an ER competitive binding assay and in a gene expression assay employing transfected MCF-7 cells. The in vivo assays undertaken in that study included a uterine wet weight assay, and a vaginal cell cornification assay, the endpoints of which are controlled by estrogenic stimuli. Rats were dosed orally with high concentrations of phthalate (20, 200, and 2000 mg/kg). Despite BBP, DBP, and DHP displaying definitive interaction with the estrogen receptor in vitro, none of the phthalates tested were able to induce estrogenic responses in these in vivo experiments (Zacharewski et al, 1998).

Knudsen and Pottinger (1999) tested DEP, DAP, DBP, BBP, and DEHP in a rainbow trout estrogen receptor binding assay. All were active, although the responses were less than 30% of the maximum, and the response curves were not parallel to that produced by the positive control, E2. The same chemicals were tested for affinity for the rainbow trout testosterone and cortisol receptors, but none were found to interact with either of these receptors.

3.1.2.2 Estrogenic activity of phthalates in vivo

The relatively recent realisation that some phthalates are weakly estrogenic in vitro has obviously raised concerns that they may also be estrogenic in vivo. Data discussing the in vivo estrogenicity (or lack of it) of phthalates are scarce, but have recently begun to emerge. The reason for the slow appearance of such data is the length of time required to undertake sound in vivo studies, together with their expense, and the lag time prior to such data reaching the public domain. Therefore, from the first reported incidence of estrogenicity (in vitro) in 1995 (Jobling et al, 1995), to date only a few articles have been published where the aim of the study was to assess estrogenic effects of phthalates in vivo. The following discussion includes some reports of effects which may not have been specifically estrogenically stimulated; however, the papers were written with endocrine disruption in mind.

The first documented evidence of apparent estrogenicity of a phthalate in vivo was published by Sharpe et al (1995). Adult female Wistar rats were dosed with BBP via drinking water, from 2 weeks prior to mating, through gestation, and until day 22 after giving birth. Subsequently, the offspring were maintained under normal conditions until the experiment was terminated at postnatal day (pnd) 90-95. These authors observed (in the male offspring) a decrease in testis weight, along with a decrease in relative testis weight and testis/kidney weight ratio, and also a decrease in daily sperm production, which was proportional to the drop in testis weight (Sharpe et al, 1995). It was clearly stated by the authors that there was no evidence of a specifically estrogenic mechanism, although it is unlikely that it was a purely toxic one, since body weight and kidney weight of the exposed animals were normal. Since the publication of these data, attempts have been made to repeat the experiments (Ashby et al, 1997a). However, there were no adverse developmental effects observed in these later studies. The reason for the different results obtained from the two studies is unclear, and hence Ashby et al (1997a) were hesitant to refute Sharpe's study, and instead described their data as "failing to confirm" that reported by Sharpe et al (1995).

There are some reports of phthalates having been tested in mammals in vivo where the endpoint was specifically dependent upon an estrogenic stimulus. Examples of such endpoints include an increase in relative uterine weight, vaginal cell cornification, and increased uterine vascular permeability. BBP was found to be inactive in the uterotrophic assay (Coldham et al, 1997). None of the phthalates tested by Milligan et al (1998) - namely DOP, DBP, BBP - stimulated an increase in uterine vascular permeability in ovariectomised mice. Zacharewski et al (1998) tested eight different phthalates (see above) in the uterine wet weight and the vaginal cell cornification assays, and found that all were inactive as estrogens. Thus, there is a consensus of opinion presently that the phthalates tested to date do not demonstrate estrogenic activity, even when administered (orally) at high doses, in short-term rodent bioassays.

3.1.4.3 Anti-androgenic activity of phthalates

The most recent hypothesis on the mechanisms behind the reproductive toxicity of phthalates is that, rather than behaving as estrogen mimics, they may in fact be having an inhibitory effect on androgen activity; that is, that they are 'anti-

androgens'. The first documented evidence for this idea was published in 1998, when Sohoni and Sumpter (1998) reported BBP to have antagonistic activity in a recombinant yeast-based androgen screen. This behaviour of phthalates will be discussed further in the following sections of this chapter.

3.2 METHODS

3.2.1 Recombinant yeast assays

The majority of phthalates used in these experiments were purchased from Greyhound Chemservice (Merseyside, UK). These were of approximately 97 - 99% purity. A total of 35 phthalate esters were assessed for estrogenic activity. Those which are used in large amounts in Europe have been discussed already, and are listed in table 3.1. However, the majority of the phthalates assayed do not have any appreciable usage in Europe, and these are listed in table 3.2. Commercial phthalate samples were also assessed for estrogenic activity in the recombinant yeast assays. These were obtained from: BP Chemicals Ltd., Hull, UK (DBP, 99.7%; DIBP, 99.6%; DEP, >99.7%; DOP, 99.9%); EXXON Chemical Ltd., Fareham, UK (DIDP, 99.9%; DINP, 99.9%); EXXON Chemical Ltd., Courbevoie, France (DTDP, purity not specified); and Monsanto Europe S.A., Louvain-la-Neuve, Belgium (BBP, >98.5%).

Phthalate metabolites were also donated by Dr. R. Bos, University of Nijmegen (MHP, MEHP, MPP, MnOP; and metabolites V, VI, and IX of DEHP - see Dirven et al, 1993); and Monsanto Europe S.A. (MBP and MBzP).

Other chemicals used in these assays were 17 β -estradiol, dihydrotestosterone, and flutamide (Sigma, Poole, Dorset). *o,p'*-Bisphenol A was obtained from DOW Europe S.A., Horgen, Switzerland.

Name	Abbr.	Mass Consumed in Europe (tonnes/annum)
Ditridecyl phthalate	DTDP	3-10,000
Diethyl phthalate	DEP	(with DMP) 10-20,000
Dimethyl phthalate	DMP	(with DEP) 10-20,000
Diisohexyl phthalate	DIHP	<2000
Diundecyl phthalate	DUP	<2000
Butyl decyl phthalate	BDcP	<1000
Butyl octyl phthalate	BOP	<1000
Dicyclohexyl phthalate	DCHP	<1000
Diethyl phthalate	DHP	not alone
Di-n-octyl phthalate	DnOP	not alone
Bis(2-n-butoxyethyl) phthalate	DBoEP	negligible
Bis[2-(2-ethoxyethoxy)ethyl] phthalate	DEoEoEP	negligible
Bis(ethoxyethyl) phthalate	DEoEP	negligible
Bis(2-ethylhexyl) hexahydrophthalate	DEHhP	negligible
Bis(2-ethylhexyl) isophthalate	DEHIP	negligible
Bis(methoxyethyl) phthalate	DMEP	negligible
Butyl cyclohexyl phthalate	BCHP	negligible
Butyl 2-ethylhexyl phthalate	BEHP	negligible
Butyl isodecyl phthalate	BiDP	negligible
Diamyl phthalate	DPeP	negligible
Dimethyl isophthalate	DMIP	negligible
Diethyl isophthalate	DOIP	negligible
Diphenyl phthalate	DPhP	negligible
2-Ethylhexyl isodecyl phthalate	EHIDP	negligible
Hexyl decyl phthalate	HDP	negligible
Hexyl 2-ethylhexyl phthalate	HEHP	negligible
Isodecyl tridecyl phthalate	IDTDP	negligible
Octyl isodecyl phthalate	OiDP	negligible
Isohexylbenzyl phthalate	IHBP	negligible

Table 3.2

Phthalates which were tested in the recombinant yeast estrogen assay, but which are not used commercially in high volumes in Europe.

The method used to assess estrogenic activity of phthalates in the recombinant yeast assay has been described in section 2.1.1. Phthalates were serially diluted over 12 steps, from 10^{-3} M, in the assay. E2, DHT, and flutamide were serially diluted from 10^{-8} M, 5×10^{-8} M, and 10^{-3} M, respectively.

The procedure for the anti-androgen assay was similar to that described for the androgen assay (see section 2.1.1), except that an appropriate concentration of DHT (2.5×10^{-9} M - sufficient to give a sub-maximal response) was added to the medium, and phthalates were serially diluted from 10^{-2} M. A chemical was considered to be anti-androgenic if its presence inhibited the colour change induced by DHT.

3.2.2 In vitro assays using mammalian breast cancer cell lines

The phthalates used in these experiments were those obtained from Greyhound Chemservice. E2 was also of the same stock as that used in the recombinant yeast assay, thus making it possible to qualitatively compare the potencies of the phthalates relative to E2 in the different assay types.

A detailed description of the method used can be found in section 2.1.2.

3.2.3 Assessment of the estrogenic activity of BBP in vivo

Since BBP was the most potent estrogenic phthalate in vitro, it was this particular phthalate which was chosen to be tested in an in vivo assay, using pair-breeding fathead minnows. The males of this species have distinct secondary sex characteristics, as can be observed in figure 3.3, which are thought to be androgen dependent.

3.2.3.1 Experimental design

Adult fathead minnows were supplied by AstraZeneca Ltd., Brixham, UK, and were exposed to BBP at a nominal concentration of $100 \mu\text{g/L}$, for a period of 3 weeks. The effect of the chemical exposure on their spawning frequency, total number of eggs spawned, and plasma vitellogenin was assessed. 4-NP, also at a nominal concentration of $100 \mu\text{g/L}$, was assessed for its effect on these reproductive parameters in the same experiment. This chemical was used because it is a known endocrine disrupting chemical. For this experiment, BBP

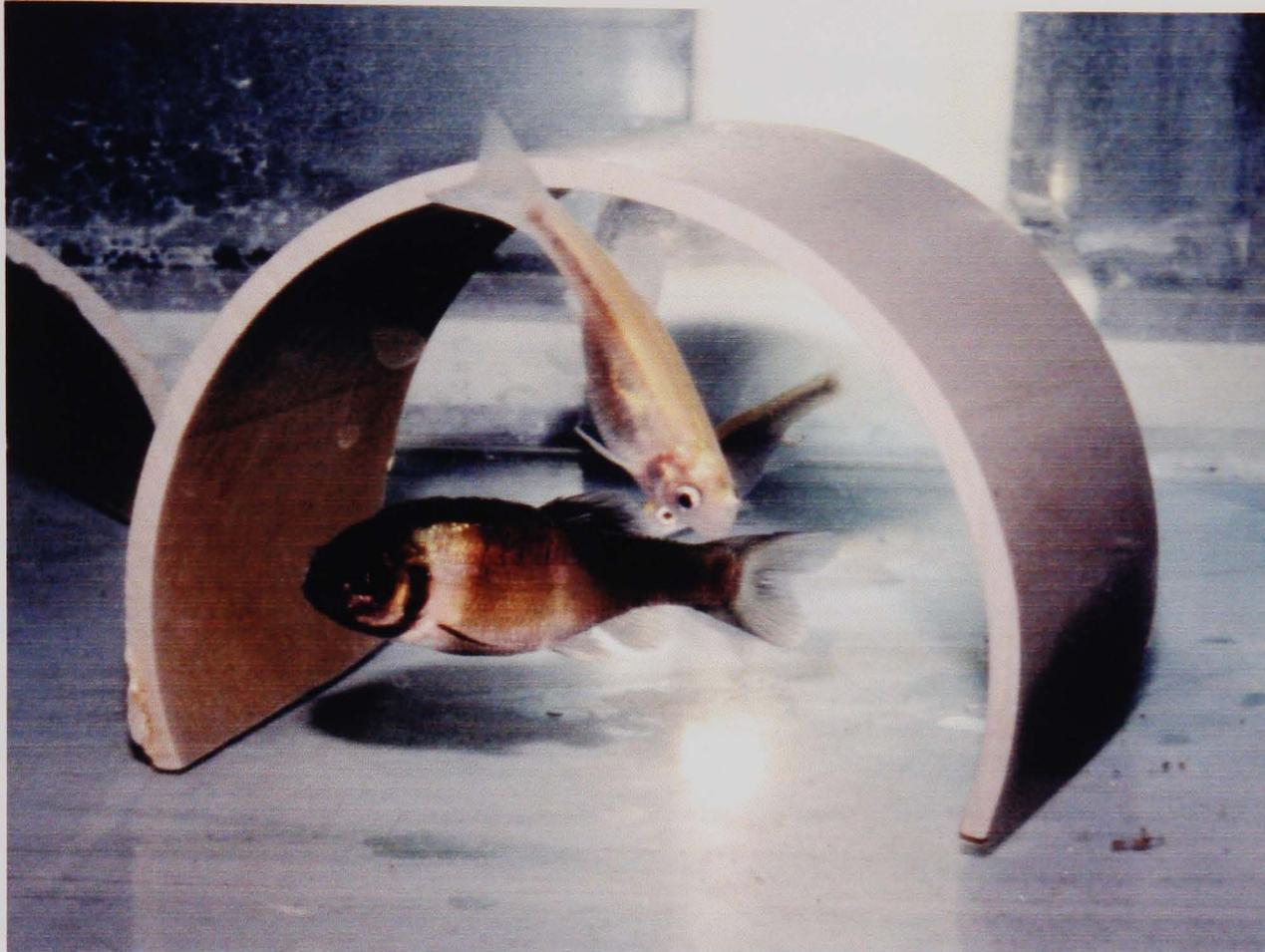


Figure 3.3

Adult male and female fathead minnows. Note that the male can be distinguished from the female by its larger body size together with its darker, stripy appearance, and characteristic fatpad which covers the top of the head and extends back over the dorsal area of the fish. The male appears to be guarding its territory – the spawning substrate (in this case a curved piece of plastic, to which eggs are attached).

was obtained from Greyhound Chemicals, and was certified as 98% pure; 4-NP (mixed isomers, 99% pure) was purchased from ACROS Organics, Leicester, UK.

A flow-through system was used in the study; tank water was replenished at a rate of 30 L/hr. Chemicals were dosed (using methanol as a carrier solvent) into mixing vessels, from stock solutions of 0.5 g/L, at a rate of 0.1 ml/min, prior to being dispensed into the tanks holding the fish. The chemicals flowed through the tanks for 10 days before any fish were added to the tanks, in order to allow the entire system to equilibrate. For each treatment, 4 pairs of fish were exposed, with 2 pairs of fish in each 30 litre tank, separated by a stainless steel grid.

Fathead minnows are relatively straightforward to work with when measurements of fecundity are required, since they stick their eggs directly onto a substrate which can be removed from the tank for egg collection. Eggs were collected and counted each day, for a 3 week period prior to exposure ("pre"), followed by a 3 week exposure period ("post"). At the end of the treatment, all fish were anaesthetised using MS-222 (Sigma, Poole, UK). The weights and lengths of the individual fish were recorded, and photographs were taken as a record of the secondary sex characteristics of the male of each pair. Blood samples were taken from the caudal sinus using 0.5 ml, 27G heparinised insulin needles, and centrifuged for 10 minutes at 14,000 rpm, the plasma was drawn off and stored at -20°C. Plasma vitellogenin concentrations were ascertained using a carp vitellogenin ELISA (Tyler et al, 1996, 1999) previously validated for use in the measurement of vitellogenin in the fathead minnow.

3.2.3.2 Chemical analysis

Water samples were taken from the treatment tanks, and from the stock bottles supplying each tank, after the 10 day equilibration period, prior to transferring the fish into the tanks. Samples were acidified with 2 ml HCl/L, and stored in the dark at 4°C. Aliquots of 100 ml were extracted onto 100 mg C18 Sep-Pak cartridges (Waters Ltd., Watford, UK), followed by elution with 2 ml methanol. The resulting eluent was reduced to approximately 0.2 ml under vacuum, and diluted with 0.2 ml water (pH 4.0); 0.2 ml of this sample was injected onto an HPLC. BBP and 4-NP were quantified by reverse phase HPLC, using a 5 µm C18 column (4.6x150 mm; Waters Nova-Pak, MA, USA). Gradient elution was carried out at 1 ml/min from methanol/water/acetic acid (70:29.9:0.1, 10 mins) to

100% methanol (20 mins). The detection instrument used was a photodiode array detector (Waters, MA, USA). Analyses of the tank water alone, or solvent treated tank water, were run to check for interference, and assess whether there was any background contamination of the water samples with either BBP or 4-NP. The limits of detection for BBP and 4-NP were 0.05 µg/L and 0.1 µg/L, respectively.

3.2.3.3 Assessment of estrogenicity of the tank water

Water samples were collected as described above, preserved with 10 ml, 38% formaldehyde/L, and stored at 4°C in darkness prior to extraction. For samples containing nominal concentrations of 100 µg 4-NP/L, 100 ml aliquots of water were extracted; for all other samples (BBP dosed tanks, control tanks) 500 ml aliquots were extracted. The extraction procedure used is described in section 2.5.2. Estrogenicity of the samples was determined using the recombinant yeast assay (2.1.1). Distilled water samples were spiked with BBP and 4-NP to give concentrations of 100 µg/L, and these were extracted using the same method.

3.2.3.4 Statistical analyses

Data were log-transformed where necessary, and analysed using Statview and SuperANOVA (Abacus Concepts Inc., Berkeley, CA). Differences between plasma vitellogenin concentrations of fathead minnows exposed to BBP and 4-NP were determined using one-way ANOVA, followed by Scheffe's post-hoc test. Differences in number of eggs spawned by fish exposed to BBP and 4-NP were determined using one-way ANOVA, followed by Fischer's PLSD.

3.3 RESULTS

3.3.1 Estrogenic activity of phthalates in a recombinant yeast assay

The estrogenic activities of the major volume usage phthalates (those exceeding 20,000 tonnes/annum in Europe) in a yeast assay are shown in figure 3.4. Of

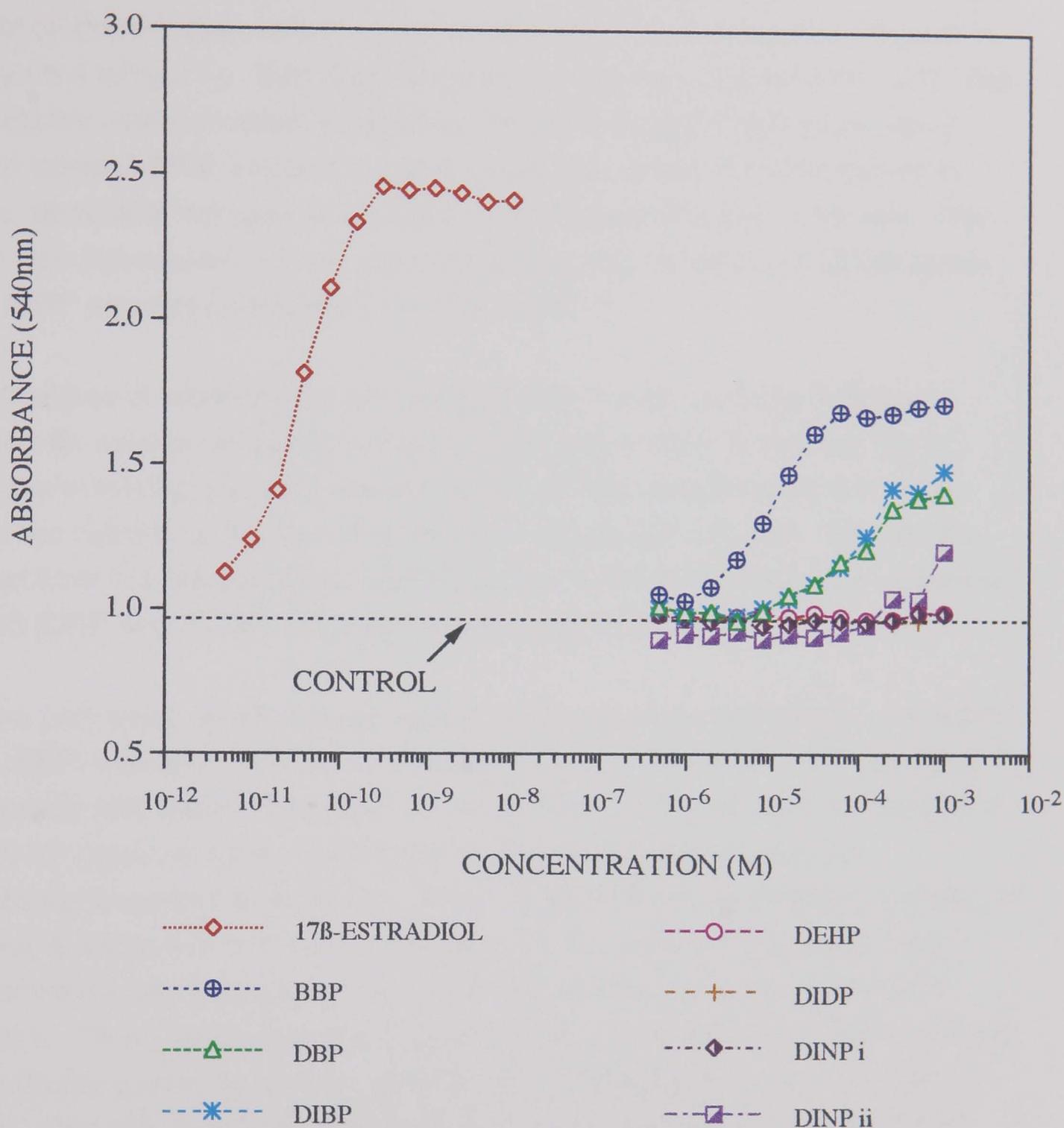


Figure 3.4

The estrogenic activity in the yeast assay of the phthalate esters used in large volumes (>20,000 tonnes/annum). Yeast cells were incubated with BBP, DBP, DIBP, DEHP, and DIDP at concentrations from 10^{-3} M to 5×10^{-7} M. DINP i represents an inactive sample of this chemical, whereas DINP ii was a sample of this phthalate which induced β -galactosidase production to a slightly greater extent than the control, in a dose-related fashion. The results obtained from the inactive chemicals (DEHP, DIDP, DINP i) cannot be distinguished individually, because they overlie each other on the baseline, illustrating the lack of response observed when these chemicals were tested in the assay.

these six major volume use phthalates, three possessed estrogenic activity (BBP, DBP and DIBP), two did not (DEHP and DIDP), and one (DINP) behaved unreproducibly in the assay. Note that the former three phthalates were the most active of all those tested, with the latter three being the most extensively used in industry (see table 3.1). Two dose-response curves were obtained for DINP, due to the slightly non-reproducible behaviour of this chemical in this yeast assay. The line labelled DINP ii shows the mean response of two standard curves in which a detectable increase in β -galactosidase production was observed. This pattern was reproduced in three separate assays, but differed in a further three, where DINP appeared completely inactive (DINP i).

The phthalates of relatively low or negligible use in Europe (table 3.2) were assessed for estrogenic activity using the yeast assay only. Relatively few of these (five in total) possessed any estrogenic activity; all others were inactive, even at the highest concentration tested (10^{-3} M) (figures 3.5, 3.6). The results obtained from the five estrogenic phthalates are illustrated in figure 3.5. Of these, only two (DEP and DTDP) are used commercially in Europe.

All of the phthalates which showed activity were very weak estrogens. The most potent, BBP, was approximately one million-fold less potent than E2, making it considerably less potent than other environmental estrogens such as bisphenol-A and 4-NP (approximately 10,000 and 9000-fold less potent than E2, respectively; Beresford et al, 2000). When chemicals are so weakly estrogenic, it is entirely feasible that it is not the chemical (in this case the phthalate) itself which is intrinsically estrogenic, but rather that an impurity in the chemical is estrogenic. Thus, before labelling a chemical as a weak estrogen, it is necessary to exclude the possibility that the chemical is contaminated with an estrogenic impurity. One way to address this issue is to test a number of preparations, of different origin, of each phthalate possessing estrogenic activity. If all preparations of a phthalate possess the same degree of estrogenic activity, then it is likely that that particular phthalate is intrinsically active, whereas if the different preparations of a phthalate possess considerably different potencies, then it is likely that the phthalate itself is not estrogenic, but that some preparations contain varying proportions of one or more contaminants which are estrogenic.

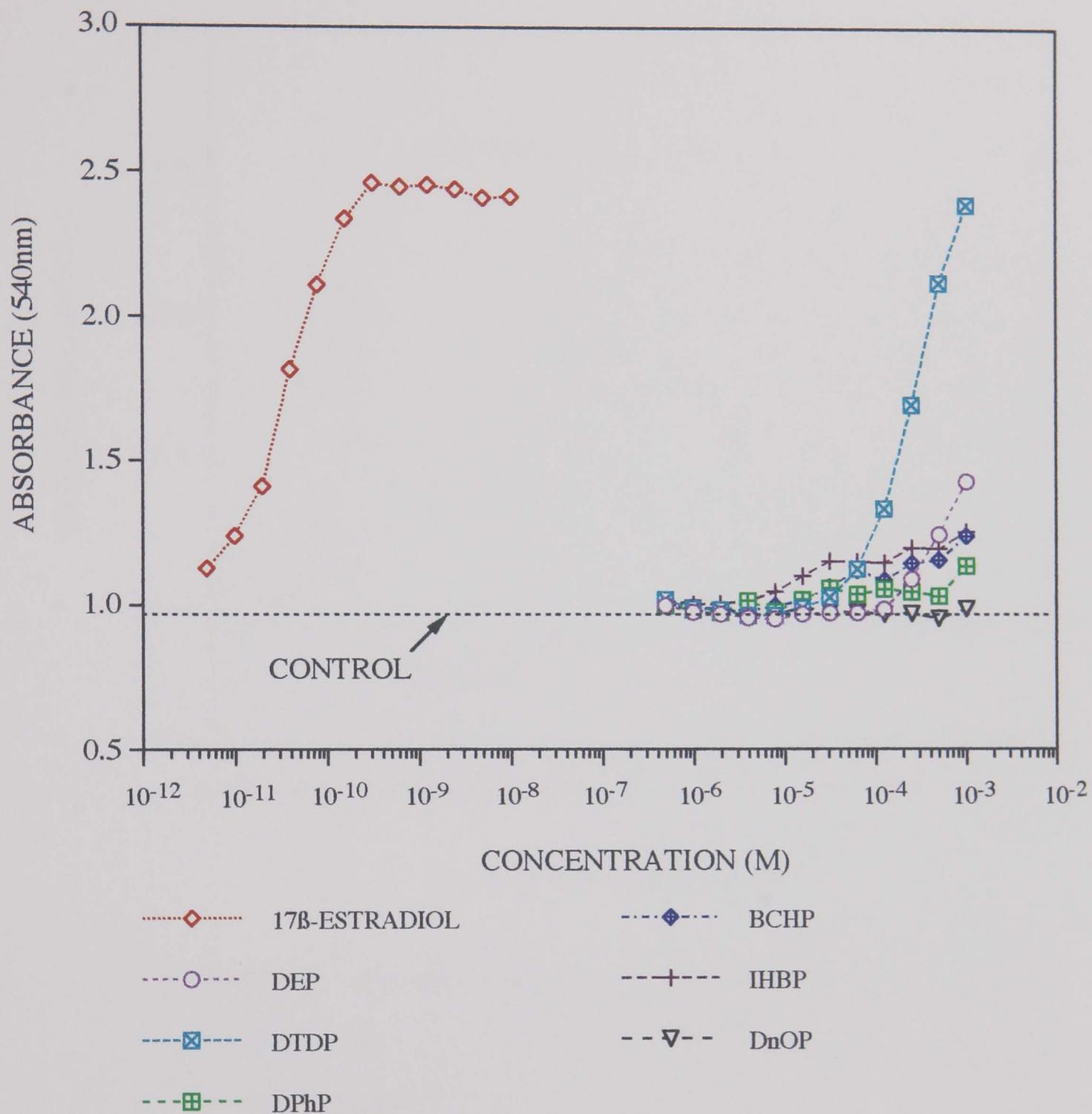


Figure 3.5

The estrogenic activity of commercially available phthalates and those of negligible use which are active in a yeast assay. DEP and DTDP are phthalates which are used commercially in Europe, albeit at volumes less than 20,000 tonnes/annum. DPhP, BCHP, and IHBP also showed dose-related estrogenic activity when tested at concentrations ranging from 10^{-3} M to 5×10^{-7} M. These latter three are of negligible commercial usage.

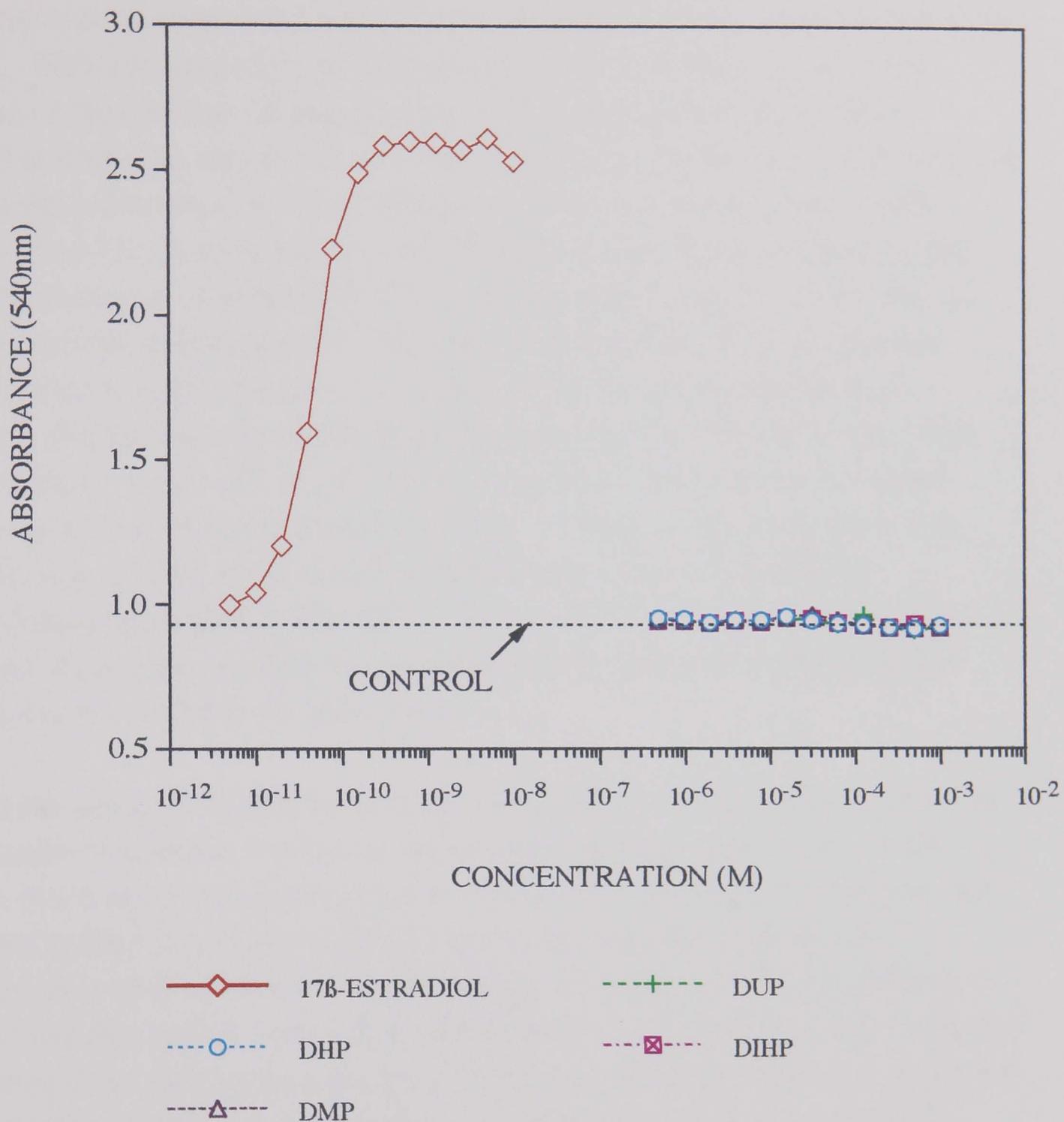


Figure 3.6

This figure portrays the lack of estrogenic activity observed in a yeast assay when the cells were incubated with certain phthalates. These were DHP, DIHP, DMP, and DUP, and were all serially diluted from 10^{-3} M. The results obtained from these phthalates cannot be individually distinguished, due to the fact that they overlaid each other on the figure, indicating the lack of deviance from the baseline.

In order to assess this possibility - that estrogenic contaminants might be present in some phthalates - commercial preparations of all the major volume usage phthalates, including DTDP and DEP, were assessed for estrogenic activity, and their potencies compared to that of their respective analytical standards (data not shown). With one exception, no differences were observed; the estrogenic activities of the commercial preparations were equivalent to those of their respective analytical standards. However, contrary to the analytical standard, the commercial preparation of DTDP failed to produce a response, even when present at 10^{-3} M. Both samples of DTDP were subsequently analysed by GC-MS. The analytical standard (the active sample) was found to contain 0.5% of the ortho-isomer of bisphenol-A. The inactive preparation of DTDP was not contaminated with this chemical. A sample of o,p'-bisphenol-A was then obtained, and its response in this yeast assay compared with that of the active DTDP sample. Figure 3.7 shows that o,p'-bisphenol-A was about 100 times more potent than the active sample of DTDP. Therefore, the presence of this chemical at just 0.5% in the DTDP sample would produce a response approximately equivalent to that seen. Thus, it is likely that this chemical (o,p'-bisphenol-A) was responsible for the weak activity observed in this phthalate sample; hence, DTDP is not estrogenic.

The results shown in figures 3.4 and 3.5 show that most of the active phthalates were unable to produce a maximal response in the yeast assay; only DTDP did so, and this was not due to the intrinsic activity of DTDP itself. For example, the response to BBP (the most estrogenic phthalate) reached a plateau at approximately 50% of the maximum response achieved with E2. To determine whether this means that most of the phthalates are only partial estrogen agonists, or whether other explanations account for the sub-maximal responses observed, a yeast assay of BBP was incubated for longer than usual, and the response monitored daily. The results (figure 3.8) show that on day 4 (the usual incubation time for our yeast assays), BBP produced a shallow dose-response curve. However, by day 6, the response was greater, and by day 13, the highest concentration of BBP had produced a near-maximal response. Note also that, whereas the dose-response curve to E2 moved approximately 4-fold to the left between days 4 and 13 (i.e. the yeast assay became more sensitive), that for BBP moved considerably further. Thus, the potency of BBP increased somewhat with time.

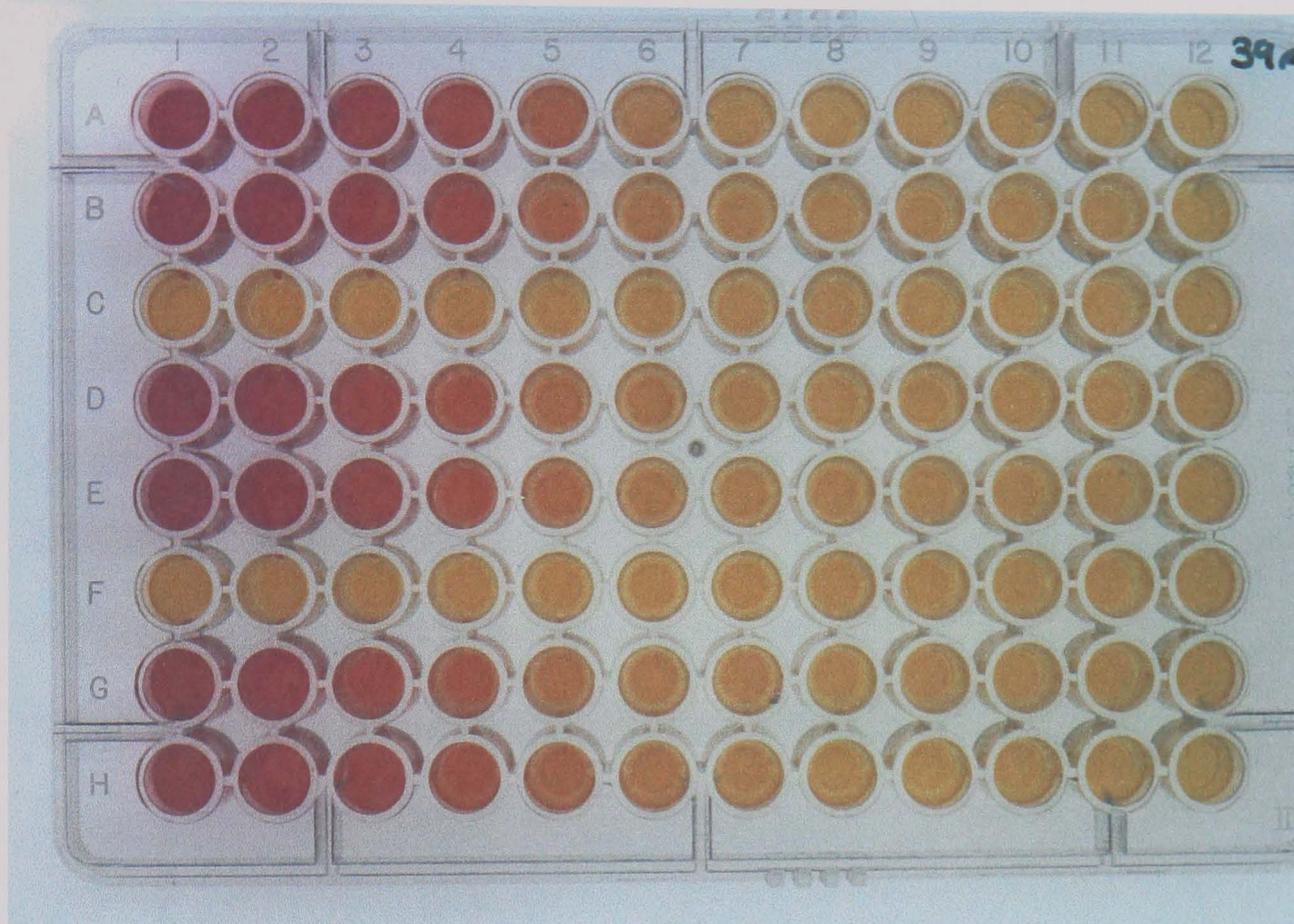


Figure 3.7

The activity of bisphenol-A (rows A and B), o,p'-bisphenol-A (rows D and E), and DTDP (rows G and H) in a recombinant yeast assay. Bisphenol-A and the ortho-para isomer of this chemical were serially diluted (left to right) from 10^{-5} M. DTDP was serially diluted from 10^{-3} M. Rows C and F are controls; $10 \mu\text{l}$ of ethanol (the carrier) was added to each of these wells.

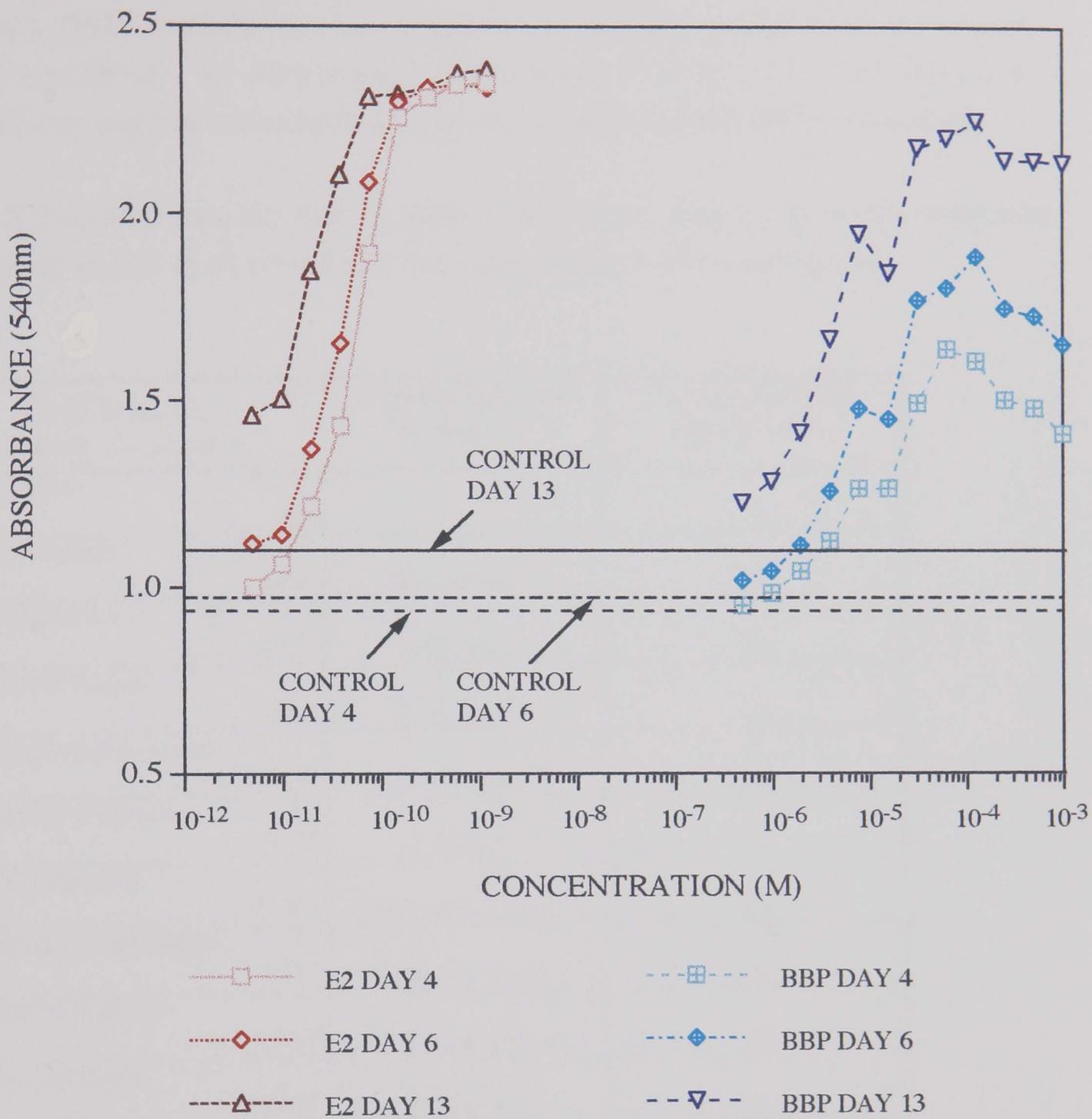


Figure 3.8

Development of the response to BBP over time in the yeast estrogen assay.

The phthalate metabolites tested included derivatives of the most abundant phthalate, DEHP, namely MEHP, and metabolites V, VI, and IX (Dirven et al, 1993); MBzP and MBP, these being primary metabolites of the most estrogenic phthalate, BBP, and MBP is also the primary metabolite of DBP and DIBP; MHP, MnOP, and MPeP. All were serially diluted from 10^{-3} M to 4.8×10^{-7} M, and none showed any signs of estrogenic activity in the yeast screen (data not shown).

Table 3.3 summarises the relative potencies and the magnitude of the responses (compared to E2) of all phthalates that were active in the yeast screen..

CHEMICAL (name & abbr.)	Approximate Potency	% Maximum Response
17 β -Estradiol E2	1	100
Diethyl phthalate DEP	0.0000005	30
Dibutyl phthalate DBP	0.0000001	35
Diisobutyl phthalate DIBP	0.0000001	30
Butyl cyclohexyl phthalate BCHP	Not determined	20
Butyl benzyl phthalate BBP	0.000001	50
Diphenyl phthalate DPhP	Not determined	10
Isohexylbenzyl phthalate IHBP	Not determined	20
Diisononyl phthalate DINP	Not determined	15
Ditridecyl phthalate DTDP	0.0000001	95

Table 3.3

The potencies of the phthalates which were active in the recombinant yeast assay. These were calculated from data obtained on day 6 of the assay. Longer incubation times can increase the relative maximum response. Thus, the values shown here apply only to a specific set of conditions. Potencies were calculated at 25% of the maximum response (in some cases, no potency could be calculated because the maximum response did not reach 25% of that produced by E2). All data shown here were obtained using analytical standards.

3.3.2 Estrogenic activity of phthalates assessed using mammalian breast cancer cell lines

To assess whether the estrogenic responses observed in the yeast assay were reproducible in other estrogen assays, active phthalates (plus the major volume use phthalates DEHP and DIDP) were also tested for their abilities to stimulate proliferation of ZR-75 and MCF-7 cells. The results from these assays (figures 3.9 and 3.10), which are based on human breast cancer cell lines, are mostly comparable to those obtained from the yeast assay. However, DEP and DTDP failed to induce proliferation of ZR-75 cells at 10^{-5} , 10^{-6} or 10^{-7} M (figure 3.9a), whereas they had been active in the yeast assay, albeit at higher concentrations only. Using the ZR-75 cells, DINP at 10^{-5} , 10^{-6} , and 10^{-7} M induced proliferation to a significantly greater extent than the control (figure 3.9b), a result which is in contrast to our findings for this chemical using the yeast screen. Growth curves for all estrogenic phthalates (i.e. those active in the yeast assay), and for DEHP and DIDP, were obtained using MCF-7 cells. The results (figure 3.10) showed that BBP was considerably more mitogenic than any of the other phthalates. DTDP, DIBP, and DBP were approximately equivalent in activity, and all the other phthalates tested showed relatively little, if any, activity. All these results are consistent with those obtained using the yeast assay.

3.3.3 Anti-androgenic activity of phthalates in a recombinant yeast assay

Figure 3.11 shows that the major metabolites of DEHP (MEHP), DBP (MBP), and BBP (MBP and MBzP), can block the binding of dihydrotestosterone (DHT) to the androgen receptor, i.e. that these phthalate metabolites are acting in an anti-androgenic manner. Also assessed for anti-androgenic activity were the remaining high volume use phthalates DEHP, DINP, DIDP, DBP, and DIBP, together with DEP which had been found to be weakly estrogenic in the yeast estrogen assay. All of these phthalates showed some evidence of anti-androgenic activity at the highest concentrations tested ($>10^{-3}$ M); all of them, with the exception of DEP, were less potent as anti-androgens than BBP (data not shown).

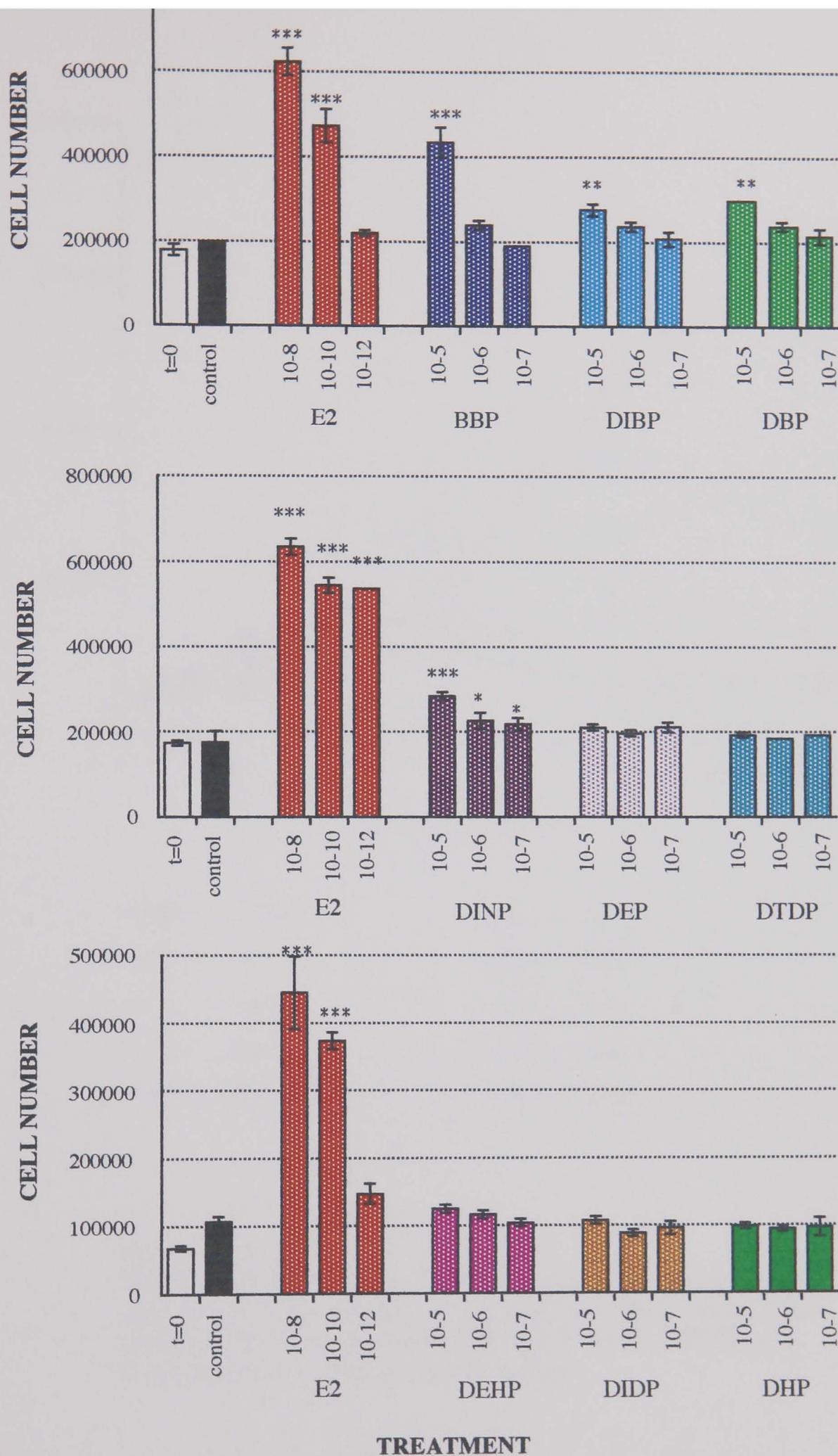


Figure 3.9

The proliferation of ZR-75 cells incubated with various phthalates is illustrated (data obtained from three separate assays). Cell numbers which were significantly greater than the control are denoted by * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$).

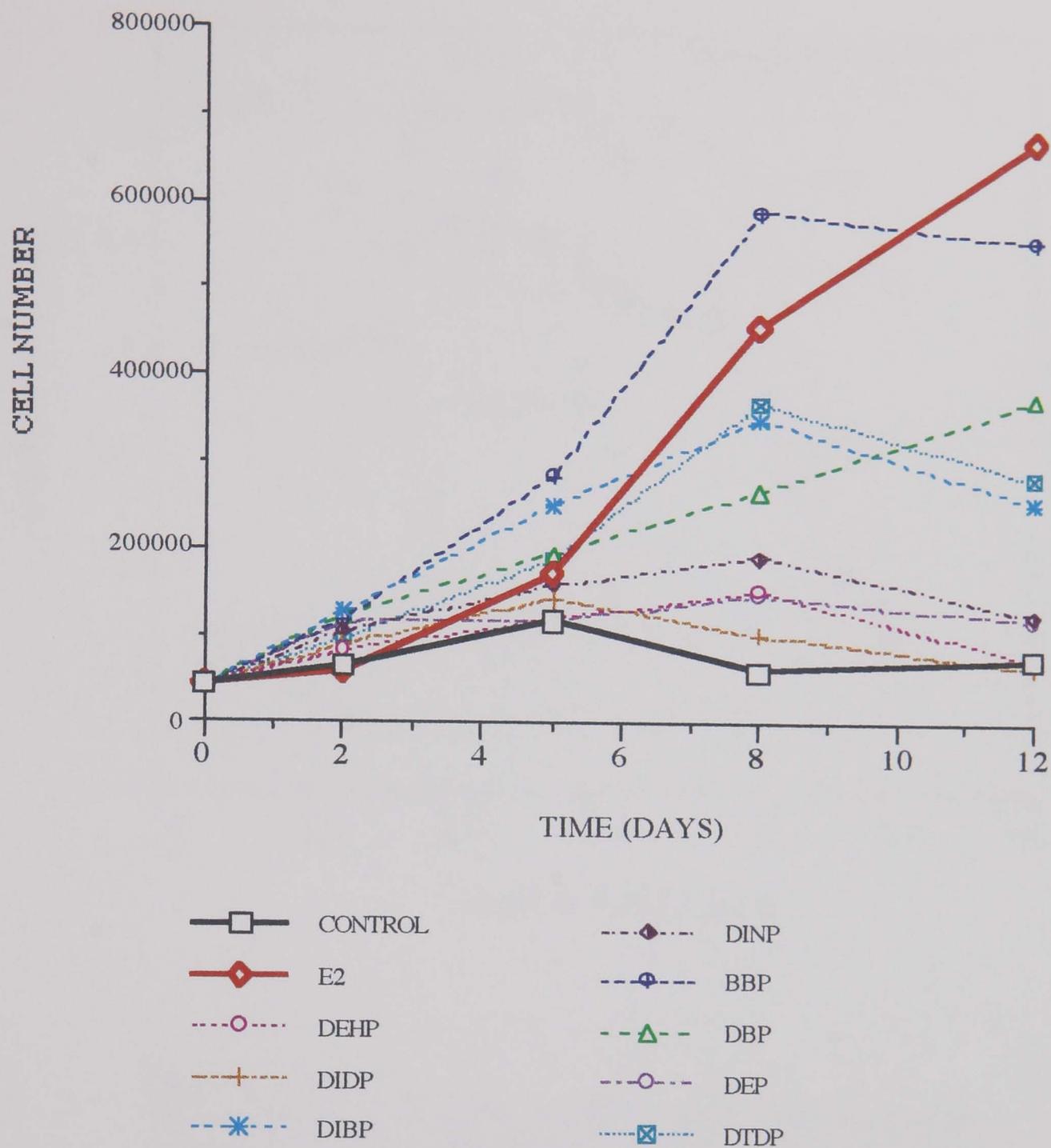


Figure 3.10

This figure depicts the proliferation of MCF-7 cells incubated with 10^{-8} M 17β -estradiol, 0.1% ethanol (control), or 10^{-5} M of DEHP, DIDP, DIBP, DINP, BBP, DBP, DEP, DTDP, and monitored over a time period of 12 days.

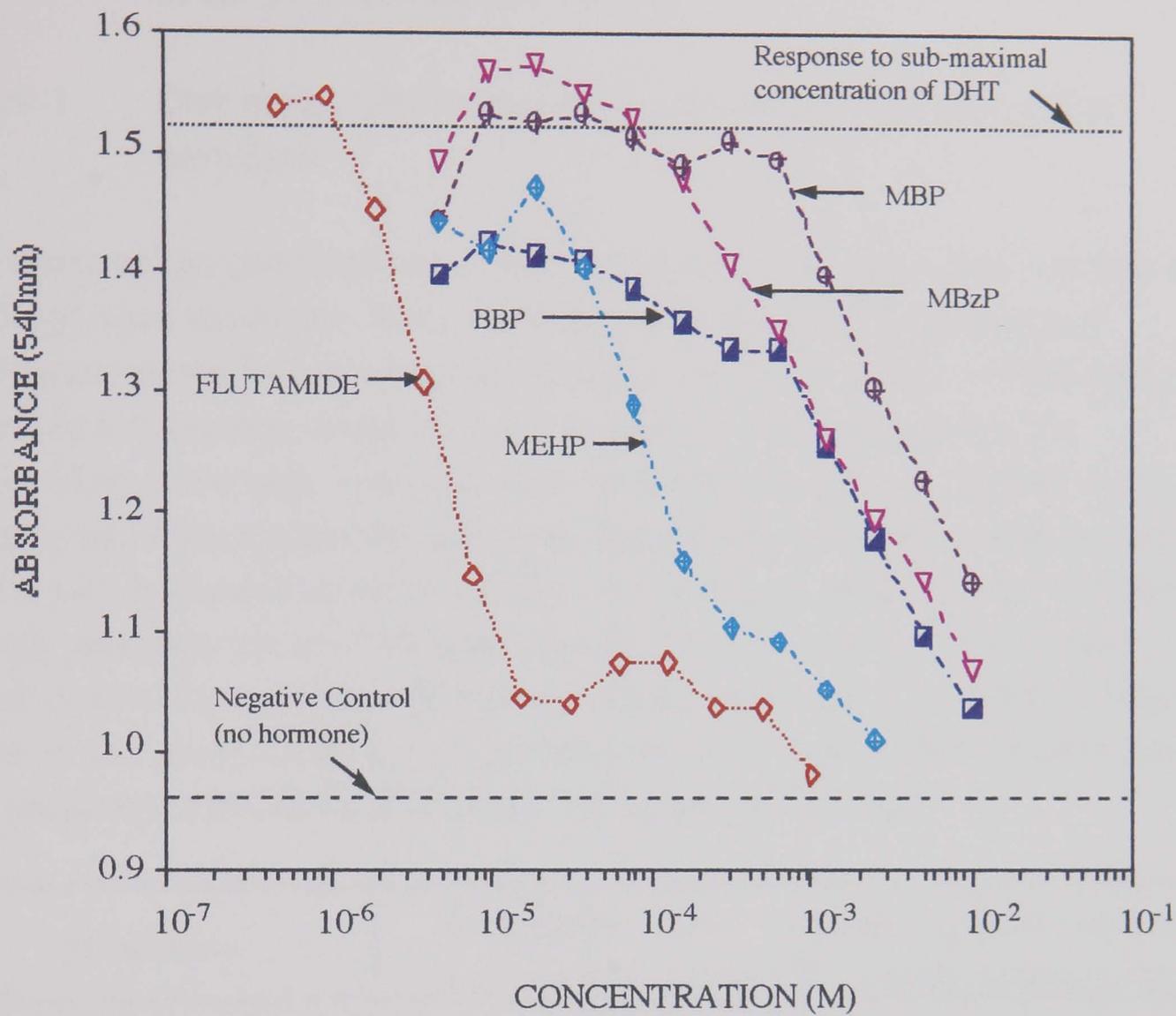


Figure 3.11

The anti-androgenic activities of BBP, MBP, MBzP, and MEHP. Each chemical was serially diluted from 10⁻² M, and compared to the activity of the clinical anti-androgen flutamide, which was serially diluted from 10⁻³ M. Anti-androgenic activity was detected using a recombinant yeast-based assay. The figure illustrates the ability of these chemicals to block the normal androgenic response to DHT.

3.3.4 Assessment of the estrogenic activity of BBP in vivo in fathead minnows

3.3.4.1 Chemistry and estrogenic activity of the tank water samples

The mean actual concentration of BBP measured in the tank water samples was 75.5 µg/L (see table 3.4). Note, however, that this was the concentration measured before the fish were transferred to the tanks (water samples were only collected for chemical analysis at one timepoint - this being prior to the introduction of the fish). It is likely that the actual concentration of BBP in the water would have decreased following deployment of the fish, due to absorption by the fish, and degradation processes. It is also true, however, that the stocking density was fairly low in these tanks (4 fish per 30 litre tank), and this factor, together with the rapid replenishment of dosed water (i.e. a complete change of water in one hour), might lead us to expect that the drop in concentration due to the presence of the fish would not be especially pronounced.

Treatment	Nominal tank water concentration (µg/L)	Mean measured tank water concentration (µg/L)
Control	0	0
Solvent Control	0	0
Butylbenzyl phthalate	100	75.5
4-nonylphenol	100	71

Table 3.4

Mean measured concentrations of BBP and 4-NP in the treatment tanks (n=2 tanks) prior to the introduction of the fish. Note that the detection limits for BBP and 4-NP were 0.05 µg/L and 0.1 µg/L, respectively; the concentrations of BBP and 4-NP measured in the control tanks were below these detection limits.

The data from the yeast estrogen assay performed on these samples are shown in figure 3.12. Water samples taken from the tanks dosed with BBP elicited a 'partial' dose-response curve, which is characteristic of that observed with BBP in this (and other) assays (see section 3.3.1). This would suggest that the estrogenic activity observed is due to the presence of BBP in these samples.

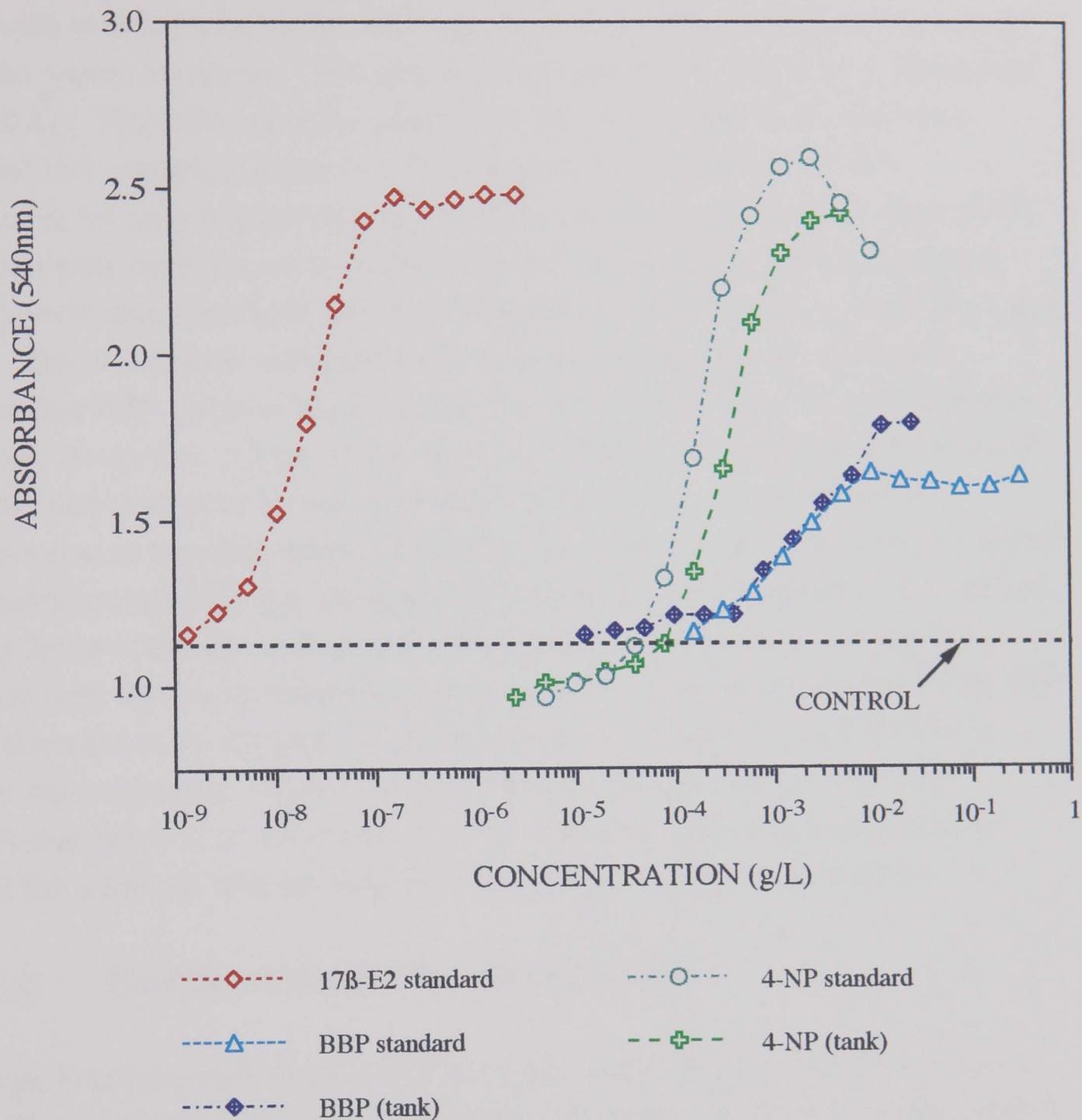


Figure 3.12

The estrogenic activities of the tank water samples compared with that of chemical standards. The water samples were concentrated during the extraction process (the BBP samples were concentrated x 2000, and the 4-NP samples x 400), and the curves have been plotted according to their nominal concentration times the concentration factor (i.e. the nominal concentration of the final concentrated sample). This means that, were the actual concentrations equal to the nominal concentrations, the response curves should overlay those of the standard chemicals.

Clearly, this is not conclusive, but it is useful in cases such as these to be able to distinguish between chemicals with distinct behavioural patterns. Likewise, 4-NP exhibits a pattern known to us as 'creeping' in the yeast assay. This results in the blank wells to either side of the 4-NP standard curve also demonstrating a very clear estrogenic response. This phenomenon has been discussed in Beresford et al (2000). The water samples taken from the tanks dosed with 4-NP also exhibited this 'creeping' behaviour, thus suggesting that the chemical responsible for inducing estrogenic activity in the yeast assay was indeed 4-NP. Water samples from the control tanks showed no estrogenic activity (data not shown), indicating that there was no estrogenic contaminant inherent in the water supply. Since the water samples were concentrated during the extraction process (the BBP samples were concentrated 2000-fold, and the 4-NP samples 400-fold), the curves in figure 3.12 have been plotted according to their nominal concentration multiplied by these concentration factors (i.e. the nominal concentration of the final concentrated sample). This means that, were the actual concentrations equal to the nominal concentrations, the response curves should overlay those of the appropriate standard curves. In actual fact, the curve obtained from the water samples dosed with 4-NP is displaced slightly to the right of the standard curve for 4-NP, implying that there is slightly less 4-NP in the sample than expected. The curve from the water sample dosed with BBP actually overlies that of the standard curve, implying that the concentration of BBP in the water sample equates with that of the nominal concentration.

3.3.4.2 Plasma vitellogenin concentrations

Vitellogenin concentrations of both male and female fish are depicted in figure 3.13. This indicates a clear and significant increase in vitellogenin in the plasma of the male fish exposed to 100 µg 4-NP/L (the concentration increased about 5000-fold). There was also a slight, but nonetheless significant, increase in vitellogenin concentration in the female fish exposed to the same treatment. Conversely, there was no increase in plasma vitellogenin (compared to the control) in either the males or the females exposed to 100 µg BBP/L.

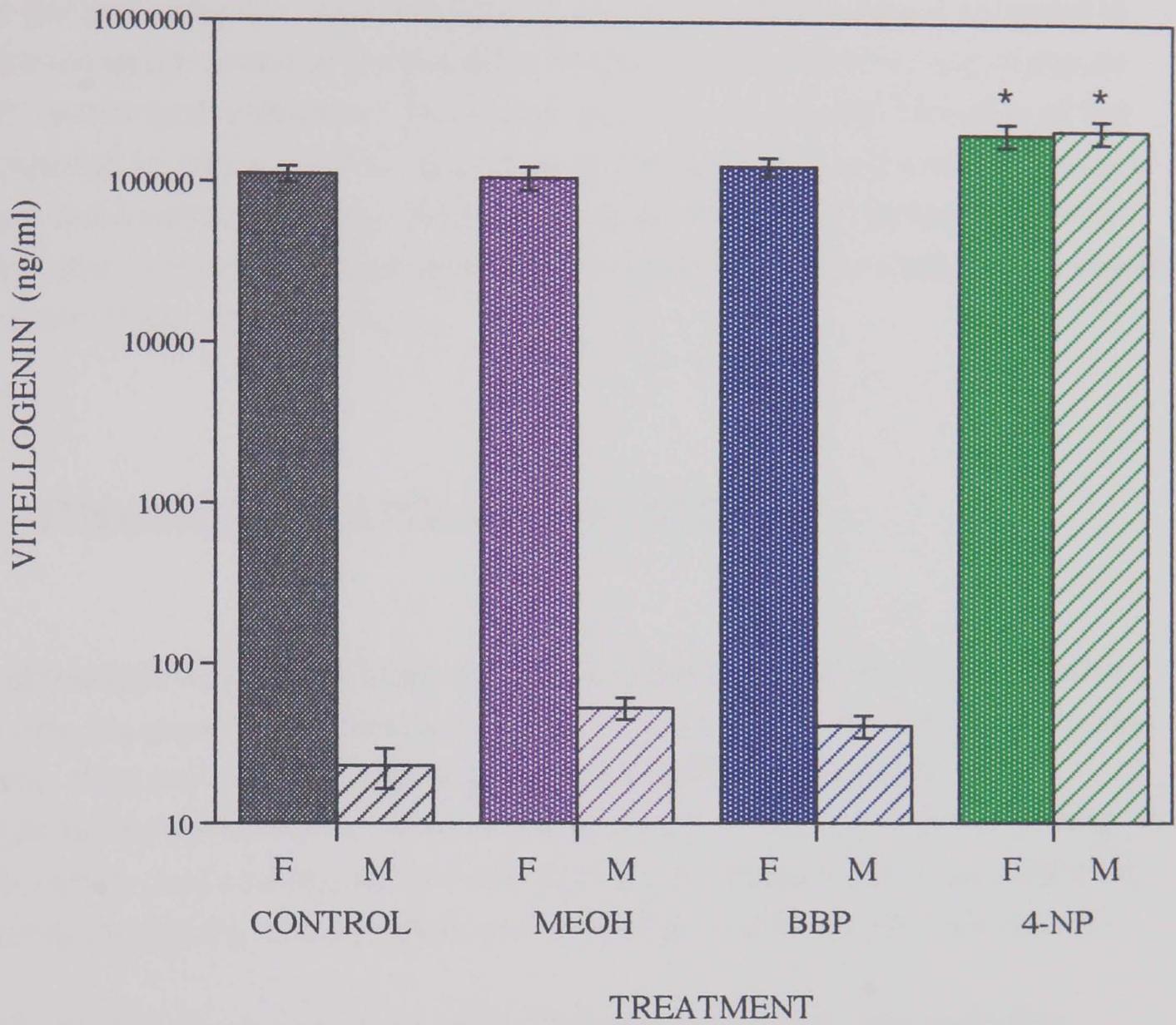


Figure 3.13

Vitellogenin concentrations in the plasma of mature fathead minnows exposed to 4-NP and BBP at 100 $\mu\text{g/L}$. 'F' denotes the female fish, and 'M' the males. Significant differences ($p < 0.05$) are shown by an asterisk.

3.3.4.3 Fecundity

The total number of eggs spawned by the fish in each treatment (expressed as a mean per pair of fish) is shown in figure 3.14. The number of eggs spawned in the pre-exposure period is compared to the number spawned during exposure ('post') within each treatment. There was no effect on absolute fecundity of the fish exposed to 100 µg BBP/L. Exposure to 100 µg 4-NP/L reduced the number of eggs spawned significantly; indeed, the fish in these tanks almost ceased to spawn (after a period of seven days exposure, two of the four pairs did not spawn for the remainder of the study).

3.4 *DISCUSSION AND CONCLUSIONS*

The phthalates which have been studied here are used by industry in variable amounts, the greatest of which is for DEHP, at up to 500,000 t/annum in Western Europe. The world-wide production of another class of chemicals, the alkylphenol polyethoxylates, was 360,000 t/annum in the late eighties (Ahel et al, 1993), which puts into perspective the large scale usage of phthalate esters as industrial chemicals, and therefore their potential environmental importance.

In terms of their estrogenic behaviour, it seems that those requiring further scrutiny include: a) the shorter chain phthalates, namely BBP, DBP, and DIBP, which are used by industry in smaller quantities (Table 3.1), but are more estrogenically active; and b) the longer chain phthalate DINP, which although extremely weakly estrogenic in vitro, is used in large quantities (up to 200,000 t/annum in Europe). The estrogenic behaviour of the phthalates in these assays compares favourably to that previously reported. For example, the potency of BBP (approximately 1 million-fold less potent than estradiol in a yeast assay) was similar to that reported by Sonnenschein et al (1995) and Soto et al (1995) in the E-Screen assay (3 million-fold less potent than estradiol), and the relative potencies of the phthalates reported to be estrogenic by Jobling et al (1995) corresponds to that observed in the yeast assay employed here (BBP>DBP). It must also be noted that, generally speaking, the activities of the phthalates in the recombinant yeast assay were reproduced in the assays based on mammalian

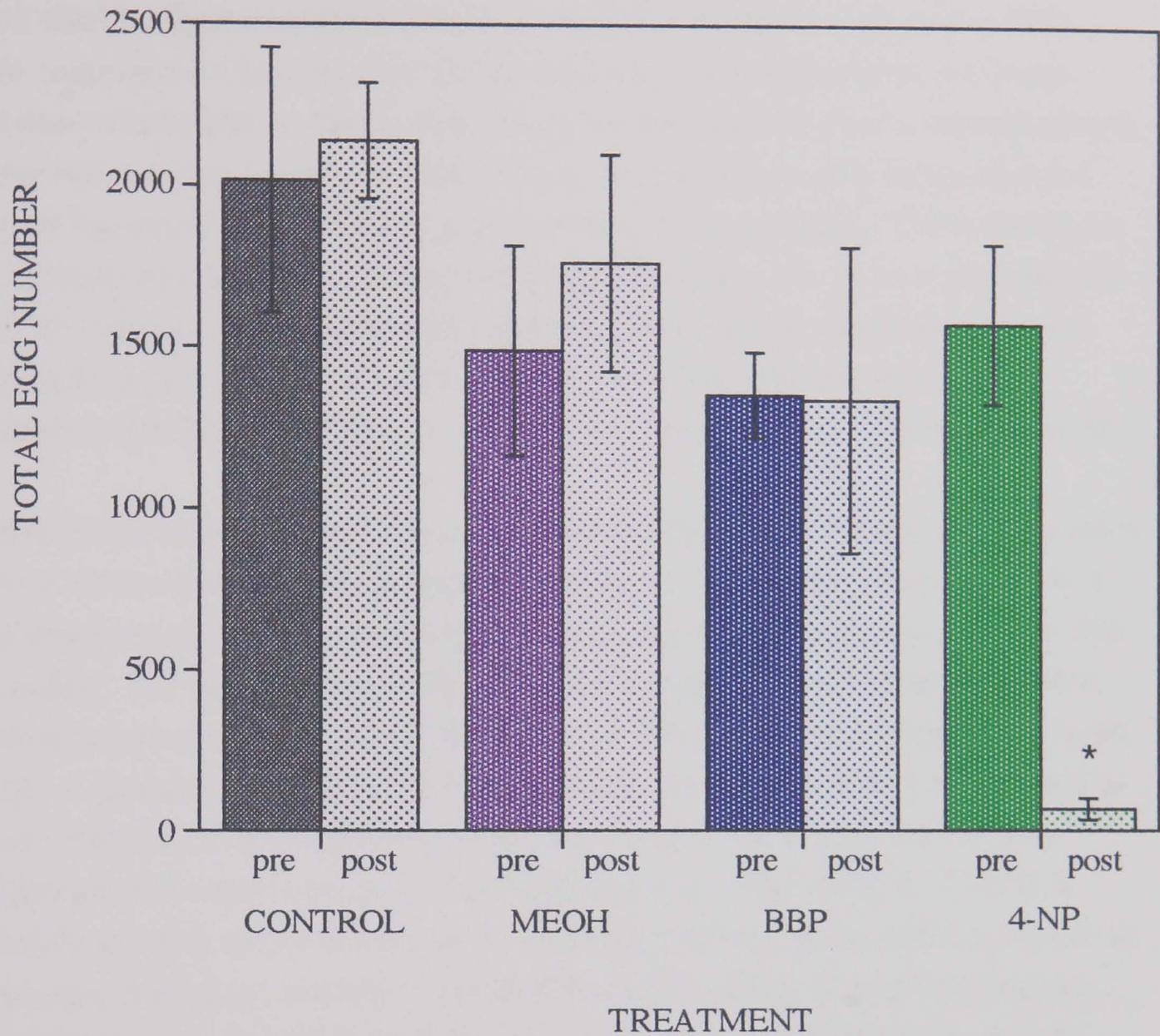


Figure 3.14

Total number of eggs spawned by fathead minnows exposed to BBP and 4-NP at 100 $\mu\text{g/L}$. Data are shown for a 3 week pre-exposure period, followed by a 3 week exposure period. * denotes a significant difference ($p < 0.05$) between the total number of eggs spawned in the pre and post experimental periods within that treatment group.

cells, thus inferring that these are real estrogenic effects, and not artefactual. There were occasional discrepancies between the results of these assays; for example, DTDP and DEP were not found to be mitogenic in the ZR-75 cell line, but had shown slight mitogenic activity in the MCF-7 assay, and produced a positive response in the recombinant yeast assay. The yeast cells are more robust than mammalian cells, and so could be exposed to higher concentrations of phthalates with no adverse effects, hence accounting for the observation of activity at the higher concentrations applied in the yeast assay. The reasons for any discrepancies between the two mammalian assays are unclear, but may be a result of the enhanced proliferation of the MCF-7 cell line in the presence of growth factors (the identity of which are not known) as well as estrogens, compared to the ZR-75 cell line, in which proliferation is more estrogen specific.

All active chemicals, however potent, are said to be active because they cause a response above that of the baseline. However, for all active phthalates, only a partial dose-response was observed after the usual incubation time used in the yeast assay. For example, for DINP, the most used of all the active phthalates, the maximum response was just 15 percent of the maximum response obtained with E2. A possible explanation for results such as these, which suggest partial agonistic behaviour of the phthalates, is that these chemicals were not fully solubilised in the water-based medium employed in these assays. This is a situation frequently encountered when applying highly organic compounds to in vitro assays, and is an entirely feasible explanation since, generally speaking, the solubility values for phthalates are lower than the concentrations used in these trials. Thus it is plausible that some of the phthalates tested are actually more potent than appears to be the case. However, it must be noted that the test chemicals were added to the medium of the mammalian cell assays in ethanol, thus leading to greater homogeneity throughout, and still only a partial response was observed. Conversely, contamination of a chemical with an estrogenic compound can imply a weak estrogenicity of the substance in question, when it is in fact the contaminant that is generating the observed response, and the chemical under investigation is not estrogenically active. This phenomenon was detected in the case of DTDP, where the weakly estrogenic preparation was found to be contaminated with the ortho-isomer of bisphenol-A. Hence, caution must be applied when labelling a chemical a weak estrogen, particularly if the chemical is not pure (which is usually the case, especially with industrial chemicals).

Blom et al (1998) reported that DEHP was estrogenically active, and as strongly so as 4-NP (a chemical widely accepted to be a weak xenoestrogen), in an in vitro MCF-7 cell proliferation assay. This result was unusual in that, in previous reports where DEHP has been found to induce a response greater than that of the control, this has been extremely slight, so much so that it has been equivocal. Further, any phthalates which have been tested in any in vitro assay in which 4-NP has also been included have been found to be far less potent than this "model" xenoestrogen (Soto et al, 1995; Petit et al, 1997; Coldham et al, 1997; Bolger et al, 1998). It is possible that Blom et al's finding of appreciable estrogenicity of DEHP is a real result, but it is also conceivable that this response was due to some form of contamination of the chemical or equipment used.

If a chemical exhibits only weak estrogenic activity in vitro, it does not necessarily follow that the effect of the same chemical, when applied to a whole organism, will be insignificant. Unfortunately, results of the nature obtained here cannot be directly extrapolated to an in vivo situation. Presently, few phthalates have been assessed for their estrogenic activity in vivo, and it will be necessary to test more of these chemicals in vivo, via different routes of exposure, before reaching conclusions. Though in vitro assays give us an idea of the potential strength of a chemical as a xenoestrogen, they cannot simulate changes to the chemical within an organism, or replicate differences in the physiological systems of individual organisms, which may influence the potency and/or bioavailability of the chemical. Metabolic processes will vary greatly depending on the route of uptake, and on the characteristics of both the chemical and the organism concerned.

The in vivo results discussed in this chapter suggest that BBP, at a concentration far higher than is generally found in the aquatic environment, is not estrogenic to fathead minnows. In addition, no effects of BBP on reproductive capability (the number of eggs produced by a spawning pair of fish) were observed. Note, however, that this study was performed over a relatively short time period (3 weeks), and that only adult fish were investigated, and not the offspring of exposed adults.

In general, there are minimal data available relating to the possible estrogenicity of phthalates in the aquatic environment. The production of the egg-yolk protein,

vitellogenin, is an estrogen-dependent process, and therefore can be used as a biomarker for estrogen exposure of wild and aquaria fish (Sumpter and Jobling, 1995). This applies particularly to males, or immature females, where basal vitellogenin concentrations are extremely low. Christiansen et al (1998) injected high doses of DBP and BBP into immature rainbow trout; DBP did not induce vitellogenin production in any of the treated fish, whereas BBP induced a very weak (3-fold) increase in vitellogenin concentration. It is not entirely clear what relevance this route of exposure has to a real environmental situation, but this was intended purely as a preliminary study to assess the activity of a chemical in a worst case scenario, presumably to be succeeded by a more realistic dosing regime. However, follow-up data have not yet been reported.

Knudsen et al (1998) monitored two alternative endpoints in juvenile rainbow trout injected with BBP at concentrations of 5 and 50 mg/kg, namely the upregulation of the ER in the liver and the induction of hepatic zona radiata proteins (Zrp). This latter protein is more sensitive to estrogens than is vitellogenin, according to Arukwe et al (1997). BBP had no significant effect on the ER binding capacity at either dose, and actually decreased Zrp levels. The reason for the decrease in Zrp levels is not known, but it was conjectured to be some kind of antagonistic effect at the receptor level. Whatever the explanation, there was no evidence of any estrogenic activity of BBP.

Thus, although there are very little data available on which to base a conclusion, it seems unlikely that phthalates will demonstrate any significant estrogenic effects in vivo in fish, especially at environmentally-relevant concentrations. Nevertheless, there is a need for a few multi-generation studies, using at least one fish species and one invertebrate species, and a variety of concentrations of the test phthalate, before robust conclusions can be drawn.

Despite phthalates not eliciting an estrogen-specific response in mammalian species in vivo, it is nonetheless clear that they are capable of disrupting the reproductive development in utero of young mammals (Ema et al, 1998; Imajima et al, 1997; Piersma et al, 1995; Wine et al, 1997). The question therefore arises, "what are the mechanisms behind this activity?" Is it possible that we have been looking in the wrong direction, and that what is actually happening is that the phthalates are having an inhibitory effect on the androgen receptor? This is without doubt an area which needs to be investigated in greater depth,

particularly given that the majority of adverse effects observed following in vivo exposure of mammalian species occur in the male offspring. As well as investigating this issue further in mammalian species, there is a need to monitor specific anti-androgenic endpoints in aquatic organisms. However, presently there is a paucity of suitable endpoints, especially in lower vertebrates and invertebrates. Thus, for example, despite having an excellent indicator of estrogen action in oviparous vertebrates such as fish and amphibians (vitellogenin), there are presently no comparable indicators of either androgenic or anti-androgenic activity. Thus, even if phthalates (or any other chemicals) did have androgenic and/or anti-androgenic activity in vivo, this would be very difficult to detect in many species, especially those, such as fish, used in aquatic toxicity tests.

Some of the primary metabolites of the phthalates have been found in this study to possess anti-androgenic characteristics. These are potentially highly significant data, when it is considered that the weak estrogenic activity of phthalates is often thought of as not constituting a risk, due to their rapid metabolism to the estrogenically inactive monoesters in vivo, and also in the environment. These data may in part explain the results of an in vivo study, describing the behaviour of DBP as similar to that of the clinical anti-androgen, flutamide (Mylchreest et al, 1998). In that study, Sprague-Dawley rats were orally administered high doses (250, 500, and 750 mg/kg/day) throughout gestation and lactation, until postnatal day 20. The spectrum of adverse reproductive effects in the male offspring included decreased anogenital distance, malformed epididymides, testicular atrophy, hypospadias, ectopic or absent testes, absent prostate gland and seminal vesicles, and diminished size of testes and seminal vesicles. In contrast, estrogen dependent endpoints in female offspring, such as vaginal opening and estrous cyclicity, were not affected. Figure 3.15 illustrates data from some of the parameters measured in that study.

A subsequent study by the same authors (Mylchreest et al, 1999) covered a shorter exposure period which encompassed gestation days 12 to 21; that is, the period during which androgen-dependent prenatal male reproductive tract development occurs. Despite widespread adverse reproductive effects in offspring of DBP-treated rats, many of which were common to both DBP and flutamide, there were some which were distinct to either one or the other of the chemicals. These included a high incidence of prostate agenesis (non-

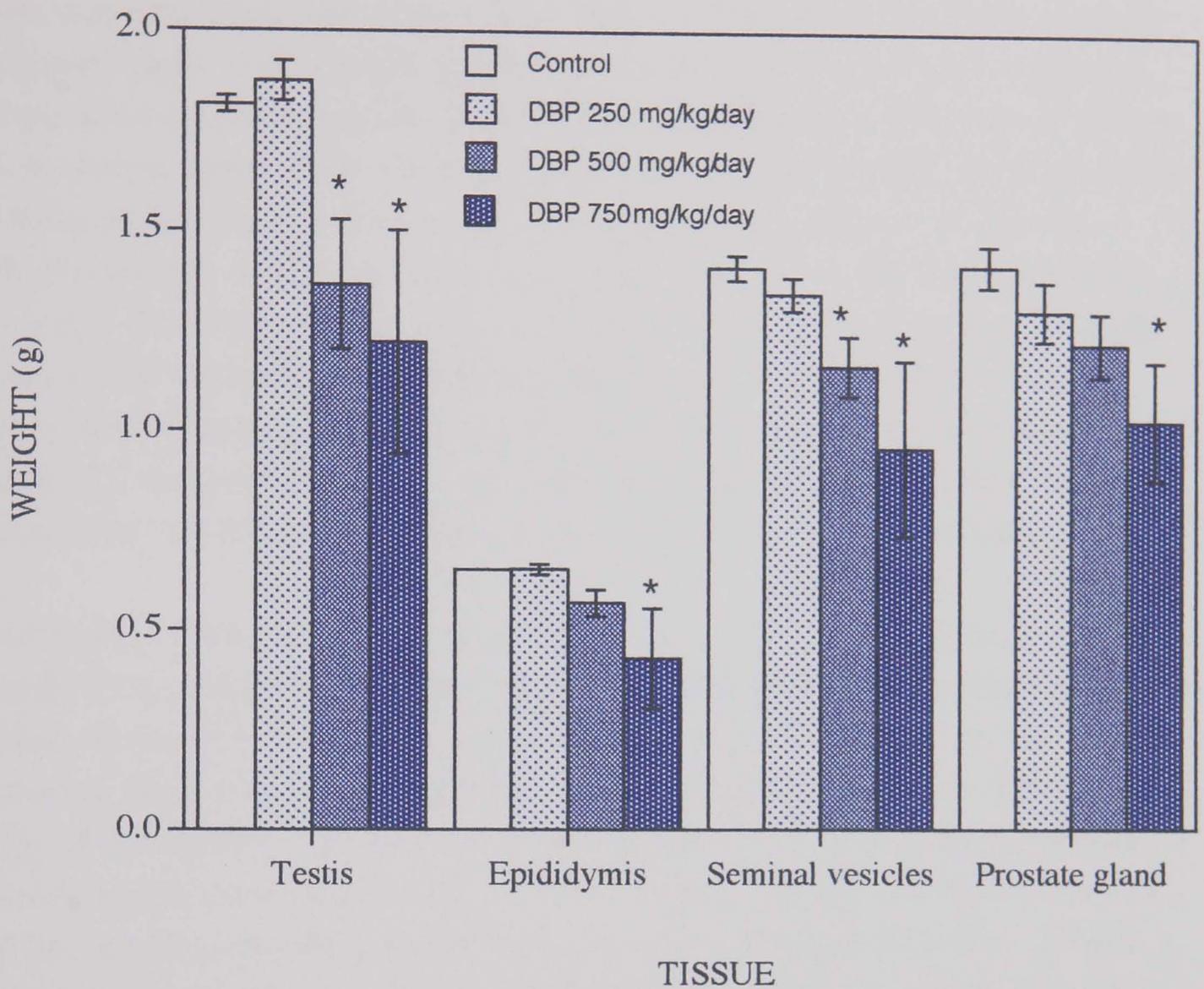


Figure 3.15

In vivo anti-androgenic effects of DBP. The data were taken from Mylchreest et al (1998), and show adverse effects of 250, 500, and 750 mg DBP/kg/day on androgen regulated endpoints in rats. The DBP was given to pregnant rats throughout gestation and lactation, through to postnatal day 20, and organ weights recorded from the male offspring. * indicates a significant difference from the control group ($p < 0.05$).

development) in flutamide-exposed offspring compared to the incidence found in DBP treated rats, and the blocking of the inguinoscrotal descent of the testis by flutamide, compared to that of the transabdominal descent caused by DBP. The authors concluded that exposure of fetuses to DBP during the sensitive window of gestation (days 12-21, covering the period of sexual differentiation in the rat), can lead to severe reproductive disruption in male offspring. Although the effects seen were considered to be mediated via an androgen-regulated pathway, it was hypothesised that the mechanism did not involve direct interaction with the androgen receptor, since the behaviour of DBP differed from that of flutamide in some ways. This interpretation was supported by the results of Gray et al (1998), who found DBP and MBP not to have an agonistic interaction with the AR in receptor binding and transcriptional activation assays. However, the data shown in figure 3.11 suggests that MBP - the metabolite of DBP likely to be found in the exposed rats - does have a receptor mediated antagonistic mechanism.

These results, demonstrating an anti-androgenic action of DBP *in vivo*, are very important for a number of reasons. Firstly, they demonstrate that, despite several decades of research on DBP, unexpected, and important, effects can still be discovered. Secondly, that despite the failure to observe estrogenic effects in females (the research was undertaken originally to see if the estrogenic effects observed *in vitro* were also manifest *in vivo*), effects indicative of another type of endocrine activity, namely anti-androgenicity, were observed in males. Thirdly, that the timing of exposure is all important; exposure produces effects only if it occurs during the relatively brief period of sexual differentiation. Subsequent research can now involve lower doses, to determine if there is a real risk at realistic levels of exposure. Such research can obviously only be conducted using *in vivo* experimental designs.

Another difficulty in estimating the environmental hazard posed by phthalate esters is the lack of data documenting the degree of exposure of humans, or wildlife, to these chemicals. The fact that phthalates are used in a wide variety of extensively employed goods is indisputable. It is also known that they can exude from these products; for example, DBP has been found to leach from dentures (Lygre et al, 1993), as has DINP from milk tubing (Wildbrett, 1973). Furtmann (1996) has suggested that the main source of phthalates are the consumer products themselves, and that there is some justification in the inference that following dumping or incineration of these products, there are considerable

phthalate emissions into the environment. The estimated total loss of phthalate esters in Western Europe has been put at 7740 t/annum, or approximately 1% of total consumption (ECPI, 1996). However, the use of such data in the analysis of environmental hazard assessment for individual chemicals is problematic, since the data are generalised, and estimates refer to total phthalates.

By far the most frequently reported phthalate, and that found at the highest concentrations in the environment, is DEHP. This is to be expected, considering its high usage and greater likelihood of persistence relative to the shorter chain phthalates. For this reason, one would also expect DIDP and DINP to be apparent in environmental samples, but reports concerning these latter phthalates are sparse. Other phthalates which have been regularly documented in food (MAFF, 1990; Page and Lacroix, 1995), air (Thuren and Larsson, 1990; Giam et al, 1980), sediments (Furtmann, 1996; Parkman and Remberger, 1995), and river water (Sheldon and Hites, 1978; Pirie et al, 1996) include the lower molecular weight phthalates, such as DMP, DEP, DBP and BBP. These are less stable as plasticisers, and therefore liable to migrate from a polymer matrix, particularly when this material is subjected to elevated temperature or surrounded by a lipophilic medium. For this reason, despite lower consumption of these phthalates compared to the higher molecular weight species, it is perhaps not surprising that they are commonly detected, albeit at very low concentrations, in environmental samples. The solubility and environmental persistence of individual phthalates is somewhat dependent upon the chain length of the phthalate concerned. For a more detailed discussion of the behaviour of phthalates in, particularly, the aquatic environment, see Furtmann, 1996.

Overall, the concentrations at which adverse effects have been reported to occur have been far higher than those which we would expect organisms to be exposed to in a real environment. It seems unlikely that the low levels of phthalates to which humans are exposed are capable of causing the trends in adverse reproductive health of males over the past few decades, such as decreased sperm counts (if true), and increased incidences of testicular cancer, cryptorchidism, and hypospadias. However, it is theoretically possible that they may be a contributory factor. The biggest problem in this area is that humans, and wildlife, are exposed simultaneously to complex mixtures of chemicals, which might (or might not) have some net adverse effect, whereas almost all

toxicology is based upon the testing of one chemical at a time on a very limited range of species. These chemicals could interact (to produce an effect) in various ways, ranging from one antagonising another, thereby reducing the anticipated effect, through to one synergizing with another, thereby increasing the anticipated effect. The phenomenon of synergism, though widely speculated upon, has not yet been proven to occur, either in vitro or in vivo (at least in research on endocrine disruption), but it is accepted that environmental estrogens at least can have additive activity when assayed together. For example, Jobling et al (1995) found BBP and DBP to have additive agonistic effects when in the presence of E2 in a transcriptional activation assay. This complicated, but very important, area of research is only just beginning.

Another factor influencing the occurrence of phthalates in the environment is their potential for persisting, and accumulating, in organic matrices. This would be expected to be high, since they are hydrophobic chemicals, and thus it might be possible to predict their environmental fate pattern based on that of other man-made organic chemicals. For example the polychlorinated biphenyls (PCBs) (Alcock et al, 1994), and 4-nonylphenol (Warhurst, 1995), bioaccumulate in organisms which are exposed to these chemicals over a period of time, and biomagnify through the food chain. However, phthalates appear to be more readily metabolised than these persistent chemicals, particularly by enzymes in the gut (Albro and Lavenhar, 1989) and in sewage treatment works, although their rate of degradation does appear to be influenced by the length of their side chain (Shelton et al, 1984; O'Grady et al, 1985). It is not known whether the yeast strain employed in my assays is capable of metabolising complex organic chemicals, although methoxychlor has shown a positive response in the recombinant yeast screen (Ashby et al, 1997b), and it has been reported that this chemical must be metabolised before it becomes estrogenically active (Ousterhout et al, 1981), thus suggesting the yeast strain is capable of metabolizing certain organic chemicals. A small number of phthalate metabolites were tested in the recombinant yeast estrogen assay. These included monobutyl phthalate (the primary metabolite of DBP and DIBP), and monobenzyl phthalate (this, or monobutyl phthalate, would be the primary metabolite of BBP). All metabolites tested were inactive in this assay, suggesting that it is the parent compound exhibiting estrogenic behaviour.

It is conceivable that the route of exposure of an organism to the phthalates is an important parameter when considering metabolism of these chemicals *in vivo*. It seems probable that they are readily metabolised in the gut, such that oral exposure would not lead to accumulation of high concentrations of these chemicals. However, there are few data available on the metabolism of this group of chemicals following inhalation or dermal exposure. It is perhaps necessary to investigate the fate of phthalates within an organism following administration via these routes, judging by the presence of these chemicals in a wide array of contact media. In addition, uptake via the gills, and hence directly into the blood system, as presumably occurs in fish, may elicit responses that other routes of exposure would not.

CONCLUSIONS

In summary, a small number of commercially available phthalate esters (BBP, DIBP, DBP, DEP, DINP) are capable of acting as extremely weak estrogens *in vitro*. The relevance of this to their environmental impact cannot be directly estimated yet, partly because comprehensive data concerning the environmental fate and behaviour of these individual phthalates are not available, and partly due to the impracticalities involved with extrapolating *in vitro* data to a whole animal situation. The phthalate most widely used by the plastic industry, and that reported on with greatest frequency, is DEHP. This phthalate did not show estrogenic activity in the assays employed in this chapter. Laboratory biodegradation studies, particularly of the shorter chain phthalates (that is, those which are the more potent xenoestrogens), might imply that concentrations in the environment as a whole, and within an organism, would not reach values high enough to be of significant danger. Although the potential exists for the above mentioned chemicals to generate adverse effects when present within an organism, the concentrations, and the conditions of exposure, required to do so are unknown. Also note that the results of *in vivo* experiments, such as those reported by Sharpe et al (1995), and Wine et al (1997), may not be due solely to the weak estrogenic activities of the particular phthalates administered, but may involve other, and possibly more important, mechanisms of action. For example, DEHP has been recognised for many years to be a reproductive toxicant (Gangolli, 1982; Gray and Gangolli, 1986; Siddiqui and Srivastava, 1992; Davis et al, 1994), yet this particular phthalate demonstrated no estrogenic behaviour in

the assays employed in this study. The potential of some of the phthalates, and their metabolites, to induce anti-androgenic effects has only recently come to light, and must also be taken into consideration. It may also transpire that it is not simply a matter of the response of a parent organism to the chemical concerned, whether exposure is acute or chronic, but that any effect may not be detected until subsequent generations. This possibility has been very clearly demonstrated by Wine et al (1997), who found that adverse reproductive effects induced by DBP in Sprague-Dawley rats were most pronounced in the second generation, although the mechanisms generating these responses are unknown.