Interference with androgen regulated tissue development by environmental contaminants that interact with steroid hormone receptors *in vitro*

by
Simon Piers Cowell
B.Sc. University of Bath, 1992
M.Sc. University of Victoria, 1996

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We accept this dissertation as conforming to the required standard

Dr. C.C. Nelson, Co-Supervisor (Department of Biology)

Dr. B.W. Glickman, Co-Supervisor (Department of Biology)

Dr. N.M. Sherwood, Departmental Member (Department of Biology)

Dr. R.F. Addison Departmental Member (Department of Biology)

Dr. J. Ausio, Outside Member (Department of Biochemistry)

Dr. S.M. Bandiera, External Examiner, (Faculty of Pharmaceutical Sciences, University of British Columbia)

Supervisors: Drs. BW Glickman and CC Nelson

Abstract

There is growing concern that the health of many species, including humans, may be threatened by an increasing burden of environmental contaminants. Many researchers around the world have made discoveries demonstrating endocrine activity in an array of contaminants to which humans and wildlife may be exposed. Although much of the focus has been upon interference with estrogen activities, there is growing evidence for interaction with thyroid, androgen and other endocrine axes. This study investigates the potential of a selection of environmental contaminants, including PCBs, pulp mill by-products, pesticides, and alkylphenols, to interfere with endocrine processes. Using tissue culture assays, we have investigated the ability of these compounds to interfere with steroid hormone signalling pathways and have focused on the underlying mechanism of androgenic effects observed through further *in vitro* assays. A transgenic mouse model was used to explore the impact of compounds of particular interest upon the development and function of androgen regulated tissues *in vivo*. Several of the test compounds possessed endocrine activity, most frequently manifest as antagonism of the androgen receptor (AR). Amongst the pesticides tested both the *o,p*'- and *p,p*'- isomers of dichlorodiphenyltrichloroethane (DDT) and *p,p*'-dichlorodiphenyldichloroethylene (DDE) were AR antagonists. Nonylphenol and a short chain ethoxylate (N-10) as well as four Aroclor mixtures and a set of congener components selected from them were also found to antagonise AR. Only hexachlorobenzene and black liquor, a pulp mill by-product, exhibited androgenic effects *in vitro*. Estrogen receptor was antagonised by β-endosulfan and *p,p*'-DDE, while *o,p*'-DDT, nonylphenol and octylphenol all acted as estrogen mimics. The glucocorticoid receptor was antagonised by β-endosulfan, while being stimulated by *o,p*'-DDT, the alkylphenol ethoxylate N-100 and PCB congener 42. Nonylphenol, Aroclor 1254 and one of the PCB congeners were tested by oral administration in mice and all produced physiological effects, with Aroclor 1254 in particular exhibiting clear anti-androgenic properties *in vivo*. Nonylphenol caused an elevation in serum thyroid hormone along with an increase in testis size and anogenital distance. In addition, the nonylphenol treatment increased the expression of an androgen responsive CAT reporter gene that is expressed specifically in the prostate. The PCB mixture Aroclor 1254 caused a decrease in prostate weight, and CAT reporter gene expression but precocious maturation of the prostate gland. In contrast the congener PCB 42 had no significant effects upon the prostate but caused increased testis weight and impacted on spermatogenesis in the epididymis. These results emphasize the sensitivity of the endocrine system and the diverse physiological functions which it regulates. They also demonstrate the ligand promiscuity of the steroid receptors and reinforce the need to evaluate the endocrine potential of substances humankind introduces into the environment.

Examiners:

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Dr. R.F. Addison Departmental Member (Department of Biology)

Dr. J. Ausio, Outside Member (Department of Biochemistry)

Dr. S.M. Bandiera, External Examiner, (Faculty of Pharmaceutical Sciences, University of British Columbia)

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List of Abbreviations

AGD	Anogenital distance
AP	Alkylphenol
APE	Alkyphenol ethoxylate
AR	Androgen receptor
ARE	Androgen response element
BCF	Bioconcentration factor
CAT	Chloramphenicol acetyltransferase
CYP	Cytochrome P450
DHT	Dihydrotestosterone
DEX	Dexamethasone
DDT	Dichlorodiphenyltrichloroethane (1,1'-bis(p-chlorophenyl)-2,2,2-trichloroethane)
DDD	Dichlorodiphenyldichloroethane
DDE	Dichlorodiphenyldichloroethylene
DH_2O	Deionised water
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
DMSO	Dimethylsulphoxide
E2	17β-estradiol
EDTA	Ethylene diamine tetra-acetic acid
E. coli	Escherichia coli
ER	Estrogen receptor
ERE	Estrogen response element
FBS	Fetal bovine serum
FSH	Follicle stimulating hormone
GR	Glucocorticoid receptor
HCB	Hexachlorobenzene
IAA	Isoamyl alcohol
LB	Luria broth
LD_{50}	50% Lethal dose
LH	Leutinising hormone

LPB	Long probasin
MR	Mineralocorticoid receptor
NP	Nonylphenol
NPE	Nonylphenol ethoxylate
OP	Octylphenol
PAH	Polyaromatic hydrocarbon
PBS	Phosphate buffered saline
PBS-T	PBS Tween (0.05%)
PCB	Polychlorinated biphenyl
PCDF	Polychlorinated dibenzofuran
PCDD	Polychlorinated dibenzodioxin
PMSF	Phenylmethylsulphonyl fluoride
ppb	Parts per billion
ppm	Parts per million
ppt	Parts per trillion
PR	Progesterone receptor
RLU	Relative luminescence units
SDS	Sodium Dodecyl Sulphate
sdH2O	Sterile, distilled water
SHBG	Steroid hormone binding globulin
SR	Steroid receptor
T	Testosterone
T3	3,5,3-L-triiodothyronine
T4	L-thyroxine
TCDD	2,3,7,8-Tetrachloro-dibenzo-p-dioxin
TR	Thyroid hormone receptor

Acknowledgements

I have received support from many places throughout the course of this study. Financial support came from the Leverhulme Trust, the Julius Schleicher memorial fund, and the NCIC; intellectual support from my supervisors and advisors, Dr. Colleen Nelson, Dr. Barry Glickman, Dr. Nancy Sherwood, Dr. Richard Addison, and Dr. Juan Ausio; and technical support from my co-workers particularly Dr. Barry Ford, Matthew Fedoruk and Cheryl Portigal. Most of all I must give credit to the person who has supported me emotionally and physically, repeatedly proof reading and compiling and encouraging me to persevere when my own motivation failed, Kathleen Barilla without whom I simply would not be here for so many reasons.

Dedication

In memory of those who brought me to my doctoral research in British Columbia but are not here to celebrate its completion. Dr. William Whish from Bath University, who as a Canadian doctoral graduate himself extolled the virtues of the great white North to an impressionable undergraduate. Dr. Joyce Moffat, who helped initiate and develop the project and recruited me as a graduate student. Mrs. Susan Cook, a friend of the Cowell family who had relocated to Vancouver and provided me with support and friendship while I first found my feet on Canadian soil. Mr. Stephen Barilla, my father-in-law who willingly gave me wise advice, support and the hand of his daughter. I know they would all be proud and delighted to see this stage of my life come to fruition.

Chapter I - Introduction

1.1 Co-ordination in Organisms

Multicellular organisms need to have mechanisms by which they can synchronize the activities of discrete tissues within the body. To achieve this they have evolved the nervous and endocrine systems, which are integrated, most notably in various centres in the brain.

The nervous system operates along a specialised network of communication pathways, the nerves, to transmit impulses very rapidly from one tissue in the organism to one or more other sites. This signal can travel by one of three methods: electrical impulse; changes in the ionic composition or ion flux of the component cells; or chemical transfer across the synapse (cell-cell connections) by neurotransmitters. Chemicals released at the synapse include amines and peptides, both of which are also functional components of the endocrine system.

Endocrine systems involve communication between sites, exclusively via chemical messages, some of which are carried by the circulatory system. Components of the endocrine system can also operate locally in either an autocrine or paracrine manner. Autocrine factors produce an effect in the cell secreting them and are often part of a regulatory feedback mechanism. Paracrine secretions affect cells or glands adjacent to the site of secretion and do not rely upon the general circulatory system to deliver them to their target sites. Endocrine systems are comprised of three components: the signalling molecules themselves, called hormones; the transport system, which typically transmits hormones from the secreting tissues through the blood stream for delivery to their target tissues; and a receptor based effector mechanism in the target tissue that implements a response to the signal.

1.1.1 Hormones

Hormones are one of a number of substances that are produced and secreted by one cell or tissue and cause a specific biological change or activity to occur in the secreting cell or in another cell or tissue located elsewhere in the body. In general hormones fall into the four chemical classes discussed below.

Peptides and Proteins

Peptides vary from two to 100 amino acids in length with most peptide hormones falling in the range of five to twenty amino acids. Since there are twenty different common amino acids in eukaryotes, peptides can carry high information content. For example a five amino acid polypeptide can generate over three million different sequences, each with a unique structure. This permits a high degree of specificity so that peptide hormones can be targeted to one or two cell or tissue types expressing a corresponding receptor. Examples of peptide hormones include insulin, chorionic gonadotropin, and luteinising hormone.

Amino Acid Derived

A number of hormones are synthesised by the modification of a single amino acid such as tyrosine or tryptophan. These peptides can be further adapted, for example by conjugation in some instances. Amongst the important hormones in this group are the thyroid hormones, triiodothyronine (T3) and thyroxine (T4), derived from tyrosine; catecholamines, dopamine and epinephrine, also derived from tyrosine; and serotonin and melatonin, which are derived from tryptophan. Amines can also function as neurotransmitters when their release is localised at nerve termini.

Lipid-Related

Once thought to be restricted to invertebrates, several lipid hormones have now been characterised in vertebrates including man. Peroxisome proliferator-activated receptors (PPAR) α, γ, and δ bind to various lipid type molecules including prostaglandins. Activation by aliphatic molecules combined with their involvement in the regulation of lipid homeostasis and inflammatory responses has made the PPARs excellent targets for therapeutic drugs. In insects, juvenile hormone (a sesquiterpenoid) controls larval-pupal metamorphosis into the adult form. In adults it performs a second function, controlling reproductive processes. Juvenile hormone is only found in insects and related groups such as crustaceans.

Steroids/ Sterols

Steroids and sterols are derived from cholesterol, which is obtained by either dietary intake or *de novo* synthesis in vertebrates. The sterol core is modified in various ways to yield a diverse array of steroid hormones that regulate an extensive selection of developmental and homeostatic events in eukaryotes. Arthropods also use steroid hormones such as ecdysone but since they lack the cholesterol synthesis pathway, specifically the ability to convert farnesyl pyrophosphate to cholesterol, they must obtain the cholesterol precursor in their diet. Plants also make extensive use of sterols including as signalling molecules.

1.1.2 Receptors

In order to produce an effect upon a target cell, a hormone binds to a corresponding protein receptor. Hormone receptors can be divided into two broad categories based upon their location; in the plasma membrane or intracellular.

Plasma Membrane Receptors

All peptide and some of the amino acid derived hormones utilise transmembrane receptors. When a hormone binds to its cognate receptor at the cellular membrane, its message is delivered into the cell by one of three pathways in order to elicit a response.

In the first pathway the receptor carries an inherent enzyme activity, such as a protein kinase, on its intracellular surface. Ligand binding regulates this activity by inducing a conformational shift in the protein structure or stimulating the clustering of receptors eliciting an intracellular signal. For example, the insulin receptor has protein tyrosine kinase activity that is activated by insulin binding to the extra cellular domain to initiate a phosphorylation cascade within the cell.

In the second case the receptor forms a channel through the membrane that can be regulated by the binding of the hormone. The opening or closing of this channel in the presence of ligand affects the influx and efflux of ions into the cell that can stimulate a cascade of events within the cell. This type of receptor is particularly common in the nervous system at synaptic junctions where the binding of neurotransmitter (e.g. dopamine) can directly initiate depolarisation of the target nerve cell perpetuating the electrical impulse.

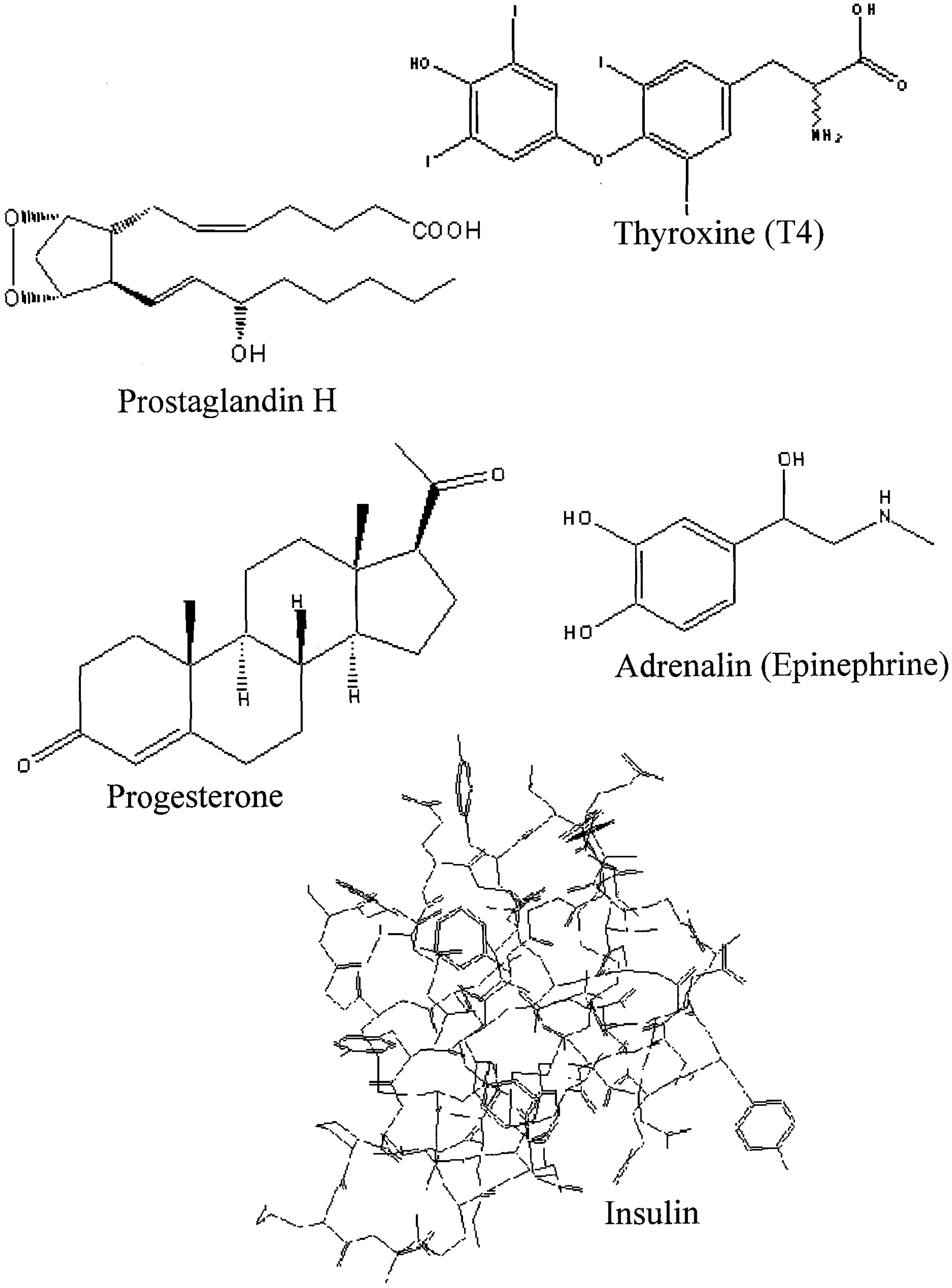


Figure 1-1: Examples of hormones from the different structural classes

In the third pathway a receptor may be linked to signalling proteins on the membrane's intracellular face. In many cases this intracellular partner is a G-protein complex, since they are activated or inactivated by cleavage of bound guanine phosphates. Binding of hormone to the receptor's extra cellular face activates the G-protein on the intracellular face of the membrane and this in turn can regulate a number of effectors to produce a cellular response. For example, the cellular receptor for glucagon functions through a G-protein complex to activate adenylate cyclase on the intracellular face of the plasma membrane in response to hormone binding.

Intracellular Receptors

If the hormone is able to cross or be transported across the plasma membrane, the hormone receptor can be located within the target cell. Typically intracellular receptors are only found for small lipophilic hormones, such as steroid and thyroid hormones as well as lipid related compounds.

There are five receptor types that bind steroid hormones: estrogen (ER; α and β), androgen (AR), glucocorticoid (GR), mineralocorticoid (MR) and progesterone (PR; A and B). These receptors are members of a growing superfamily of structurally related intracellular receptors that share the ability to bind directly to discrete DNA sequences (Figure 1-2). The thyroid hormone (TR), retinoic acid (RAR), and retinoid X (RXR) receptors form the basis of a second major sub-family of intracellular hormone receptors. In addition, there is an expanding group of so-called orphan receptors, which are identified on the basis of their structural similarity to the superfamily; for many of these, no endogenous ligand has as yet been identified. Collectively, these structurally related proteins are referred to as the nuclear hormone receptor superfamily. There have been several reports [1-6] that under certain circumstances steroid hormone receptors, particularly the estrogen receptor, may be found in or near to the cell's plasma membrane. These membrane-associated steroid receptors may represent modified forms of the classical receptors, novel proteins or may simply be an artefact of the membrane preparation methods used [6-9].

1.2 Steroid Hormones and Their Receptors

1.2.1 Structural Features of Steroid Receptors

All the members of the nuclear receptor superfamily are composed of five major functional regions (Figure 1-3A). The amino-terminal A/B region is the least conserved between the receptors and is the most variable in length; in hMR, it is over 600 amino acids while in hERα it is less than 200 amino acids long. It has been shown to encode a potent transactivation domain (AF- 1) in the steroid receptors but is almost absent in some other nuclear receptor superfamily members. Transactivation through the AF-1 region is ligand independent [10, 11].

Region C lies in the middle of the receptor proteins and shows greater than 40% homology between all superfamily members, and near complete identity between receptors for the same hormone from different species. This region is responsible for the DNA binding properties of the steroid receptors. Region C is approximately 70 amino acids in length and forms two zinc fingers, each consisting of four conserved cysteine residues chelating a zinc ion, that bind to the major groove at a specific DNA sequence. Two highly conserved short amino acid sequences, called the P- and D-boxes, determine the specificity of the receptor for its corresponding hormone specific, DNA response element (Figure 1-3B). If the P-box sequence of one steroid hormone receptor is mutated to match the sequence for another, the DNA binding specificity of the protein is correspondingly altered to match that of the acquired P-box sequence [11, 12]. The crystal structures for the DNA-bound estrogen and glucocorticoid receptor DNA-binding domains have been solved [13-15]. These findings have corroborated other experimental evidence regarding the DNA-binding specificity of these two short regions and confirmed the intimacy of the P- and D- box residues with the individual base pairs of the hormone response elements. In addition, the D-box forms an essential component of the receptor dimerisation interface, suggesting that dimerisation and DNA-binding are closely linked. Adjacent to the DNA-binding region is a short poorly conserved D-region that appears to act as a hinge, allowing movement of the carboxyl-terminal portion of the protein with respect to the DNA binding domain.

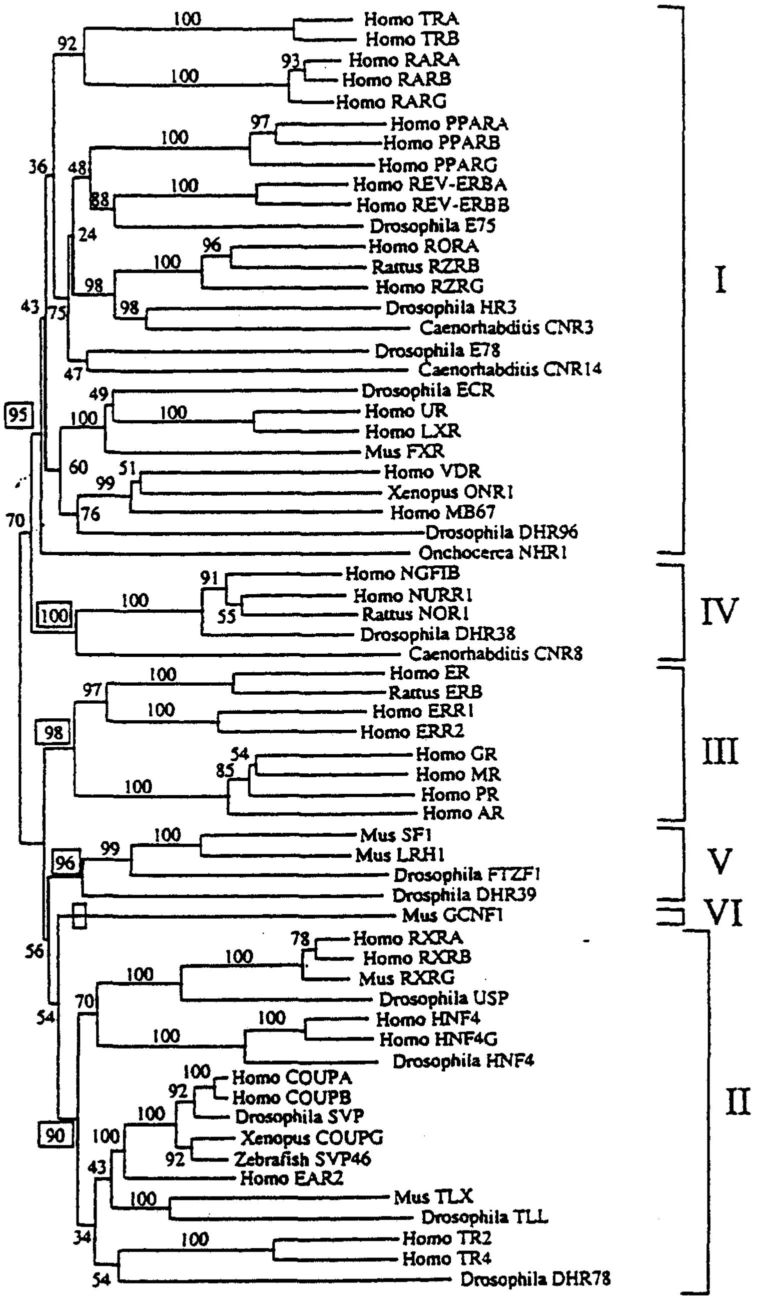
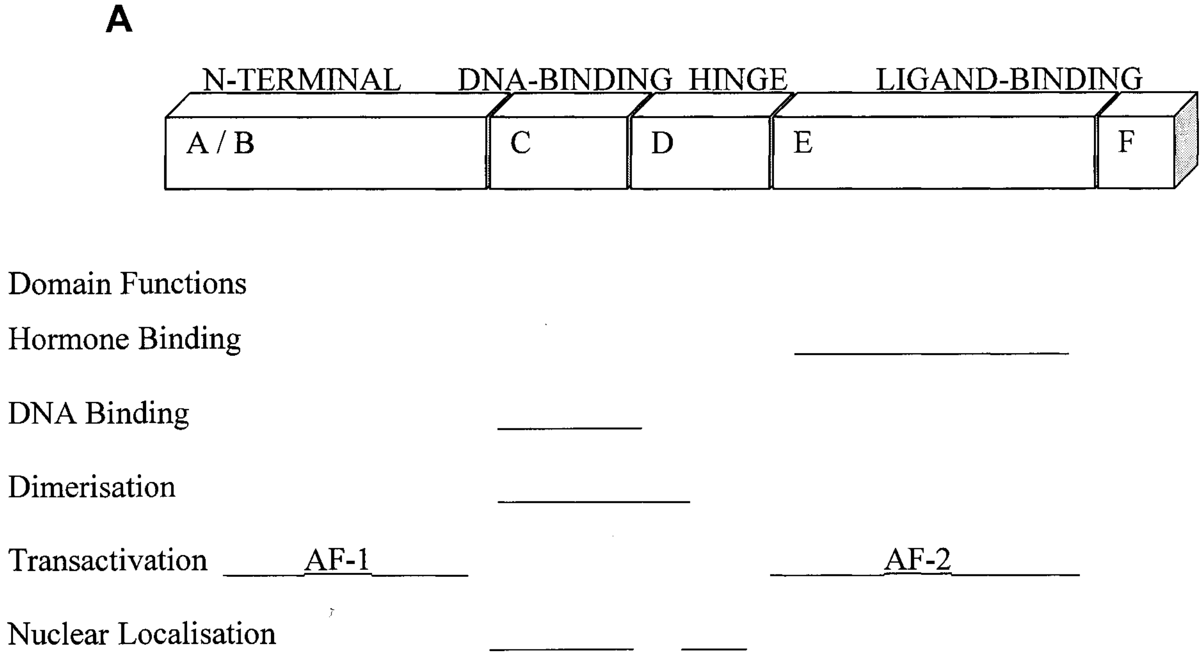


Figure 1-2: The steroid hormone receptor superfamily.

An unrooted neighbour-joining phylogenetic distance tree representing relationships amongst 63 members of the nuclear receptor superfamily. The receptors are grouped into six sub-families. From Laudet [16].

The remaining carboxyl-terminal portion of the protein contains regions E and F. These regions are not as highly conserved between different members of the nuclear receptor superfamily but exhibit high homology between receptors for a particular hormone across species. The E-region has been shown to encode the ligand-binding pocket. Hormone-dependent transactivation by steroid receptors is entirely dependent upon the activation function 2 (AF-2) domain within region E whereas the transactivation function of the A/B region (AF-1) of the steroid receptor is ligand independent. Truncation mutant receptors in which the E-region is deleted can constitutively activate steroid responsive genes although to a lesser degree than the response seen for the liganded wild type receptor [12].

For several members of the nuclear receptor superfamily including AR, the ligand-binding domain (LBD) structure in various conformations has been derived by x-ray crystallography [17]. All ligand-binding domains for nuclear receptor superfamily members have a similar structure comprising twelve α-helices. The bound ligand appears to be buried deep within the hydrophobic core of the LBD sandwiched between these helices. The biggest structural change that seems to result from ligand-binding is the reorientation of helix 12, which has been shown to contain the core of the AF-2 transactivation domain [18, 19]. In the unliganded receptor, helix 12 protrudes from the LBD, but folds in tightly upon ligand entry and makes contacts with bound ligand. In the agonist-bound conformation this produces a hydrophobic surface that attracts other coregulatory proteins [19-21]. Some antagonists may cause similar conformational shifts but the resultant conformations are less stable or fail to form the coregulator binding interface [19, 22-27]. Other structural changes in the LBD resulting from ligand binding seem to result in a general tightening of the domain to a more compact form. The E-region also contains the sequences that form interfaces for the interaction with heat shock proteins and for homodimerisation, suggesting hormone binding induced conformational change is the trigger for release of the receptor from the cytoplasmic complex [28, 29]. Region F is only present in a few of the receptor superfamily members, including the estrogen receptor, and appears to have a limited modulatory effect on certain promoters.



B

RECEPTOR	P-BOX	D-BOX	RESPONSE ELEMENT HALF SITE
TR, RAR, VDR RXR, PPAR	cEGckG	various	AGGTCA
ER	cEGckA	PATNQ	AGGTCA
GR, MR, PR	cGSckV	AGRND	AGAACA
AR	cGSckV	ASRND	AGAACA

Figure 1-3: Structural features of steroid hormone receptors.
Schematic diagram of the five major functional regions of the nuclear receptor superfamily. (B) Amino acid sequence of the P- and D-box regions of the C-domain of steroid superfamily receptors determines their DNA sequence binding specificity. ER is more homologous in this region to some non-steroid binding receptors than to the other steroid hormone binding receptors. AR is distinguished from GR, MR and PR only by a single amino acid change in the D-box region (Adapted from Tsai and O'Malley, 11).

1.2.2 Characteristics of the Individual Steroid Receptors

Estrogen Receptor (ER)

ER is the most studied member of the nuclear receptor superfamily. The classical ER (now termed ERα, see below) is a 66 kDa protein in humans. The highest affinity endogenous ligand for the receptor is 17-β-estradiol (E2) but a number of related steroidal structures are thought to play a significant role *in vivo*. Although generally viewed as the "female hormones," estrogens play a pivotal role in the development and health of both males and females of all animal species. The best-characterised roles of estrogens include regulation of the menstrual cycle, the development of the female reproductive tissues, and skeletal development and maintenance of bone mass. Estrogen response elements have been identified in the promoters for dozens of genes, underlining the diverse and potent role of these hormones. For example, estrogens have been shown to act through the ER to regulate the expression of genes encoding hormones in the hypothalamus and pituitary, which in turn regulate fertility. Estrogens have a generally stimulatory effect, increasing the synthesis and release of gonadotropin-releasing hormone and leutinising hormone [30].

As shown in Figure 1-3, the consensus DNA binding sequence of the estrogen receptor is unusual amongst the steroid hormone receptors and has greater similarity with several other non-steroid binding superfamily members. This divergence may extend further since ER shares several co-regulators with the thyroid receptor family whereas the other steroid hormone receptors may interact with a slightly different set of proteins.

In 1996 a novel estrogen receptor encoded upon a separate gene was identified [31]. This protein was called ERβ, with the α suffix assigned to the original ER identified. Characterisation of ERβ has revealed that it differs from ERα in several ways. The pattern of ERβ expression is quite different, with high levels being detected in some tissues, which express almost no ERα, such as prostate [32, 33]. There is also evidence that the ligand-binding specificity varies between the two ERs. ERβ has a lower affinity for 17-β-estradiol but seems to have higher affinity for several xenoestrogens [34, 35] and subtype selective ligands have been identified [36]. Recent evidence also suggests that recruitment of co-regulators may also differ between the two estrogen receptors [37], Finally there has been some suggestion that the DNA binding specificity of ERβ is different, although the P-box sequence is identical [38]. A subtle difference in DNA binding specificity could result in the regulation of different sub-sets of genes by the two estrogen receptor forms. There is evidence to show that a functional heterodimer can be formed between the two ERs [39] which may recruit different subsets of co-regulators. In light of some of the other discrepancies between these two receptors, such a possibility could have far reaching implications for the mechanism and regulation of estrogen responsiveness in cells.

The remaining steroid hormone receptors (AR, GR, MR, and PR) have several common features that distinguish them from the ER. They all share an identical P-box sequence and only AR differs in the D-box region by a single amino acid (Figure 1-3B) whereas the D-box of ER differs at 4 of the 5 positions. As might be expected these four receptors have all been shown to be able to bind to the same DNA response element sequence *in vitro* [40]. Furthermore these receptors share further regions of sequence homology such as the carboxyl-terminal segment of the ligand-binding domain.

Variant isoforms have been reported for all of the steroid receptors [41-46]. Unlike ER where the α and β forms are coded by two distinct genes, for the remaining four receptors it seems variation is generated by use of alternate transcriptional start sites.

Androgen Receptor (AR)

As estrogens are popularly regarded as female hormones then androgens would be their male counterparts. However, research has revealed that both sexes require a carefully regulated combination of all steroid hormones to achieve normal development and reproductive potential. The full-length human androgen receptor is a 901 amino acid protein expressed in numerous tissues, particularly testes and prostate.

Although androgen response elements (AREs) have been found within a number of genes; as with estrogen, one of the best-studied effects of androgens is on the hypothalamic-pituitary axis where they inhibit gonadotropin-releasing hormone secretion from the hypothalamus and leutinising hormone from the pituitary. Androgens are crucial to the development and maintenance of male reproductive system. Mutation of or failure to express the androgen receptor can lead to testicular feminising syndrome, where due to the resultant androgen insensitivity a genetically male embryo develops as a phenotypic female [47].

The prototypical androgen receptor ligand is testosterone. In many androgen-responsive tissues, such as the prostate, testosterone is intracellularly modified by 5α-reductase to dihydrotestosterone (DHT), which exhibits an increased affinity for the receptor and induces a greater effect on transcriptional activity [48].

Glucocorticoid Receptor (GR)

The glucocorticoid receptor is the smallest of the steroid receptors and can be detected in almost every tissue and cell type. The functions of glucocorticoids include the regulation of the immune response and energy metabolism, particularly the stimulation of glycogenogenesis. This stimulation is achieved via the activation of transcription of numerous genes involved in the synthetic pathway, including several involved in amino acid metabolism.

GR down-regulates the expression of a variety of genes involved in the development of an immune response including cytokines and their receptors as well as several enzymes. Glucocorticoids have also been implicated in the initiation of apoptosis in T-cells. Dexamethasone is a widely prescribed pharmaceutical analogue of cortisol, which is particularly effective as a modulator of inflammatory responses.

The initial cloning of the human glucocorticoid receptor (GR) revealed that although it was encoded by a single gene on chromosome 5, two different mRNAs were produced suggesting two isoforms of the receptor [45]. The predominant form, GRα, is 777-amino acids in length, whereas alternative splicing generates the 742-amino acid GRβ. Both forms are identical for the first 727 amino acid residues but in GRβ the last 50 carboxyl-terminal amino acids are replaced by an unrelated 15 amino acid sequence. It has been demonstrated that only the GRα form is able to bind glucocorticoids and that the β-isoform acts as a transcriptional repressor via competition for the response element binding site in target genes [49]. Further work has shown that differential expression of the two isoforms can be induced by proinflammatory cytokines to attenuate the immunosuppressive affects of the GRβ isoform [50].

GR is unusual amongst the steroid receptors since it exhibits many well-studied negative regulatory functions. It appears that there are two methods of repression of gene expression utilised by the receptor, both of which require the presence of ligand to be initiated. Firstly, it has been demonstrated that there are specialised response elements for the receptor termed negative glucocorticoid response elements comprising a normal glucocorticoid response element (GRE) that overlaps with a response element for a different transcription factor. Binding of GR can produce steric hindrance to the binding of the other factor [51, 52]. GR itself appears not to function as an effective transcriptional activator from these sites [53-55]. Secondly, it has been demonstrated that GR is able to interact directly with certain other transcription factors via protein-protein interactions thereby sequestering them and inhibiting transactivation by them [53, 56-58]. Although this latter form of repression by GR has been shown to be independent of DNA binding it still requires portions of the GR DNA-binding domain, which contain residues important for receptor dimerisation. Such transcriptional repression by GR depends upon the recruitment of specific coregulatory proteins [56]. Thu

Mineralocorticoid Receptor (MR)

Human MR contains 981 amino acids and is the largest of the steroid hormone receptors. Expression of the protein is restricted to a limited number of specialised tissues related to its function in the regulation of sodium transport and homeostasis. Its two isoforms, α and β, are generated by alternate splicing and appear to be expressed in approximately equal amounts [59].

MR is most closely related to GR, with 95% amino acid identity between their DNA binding domains and 60% between their ligand-binding domains, and both have identical DNA sequence specificities *in vitro* [60]. Despite these similarities the two receptors elicit very different physiological responses. This is attributed to the different tissue distributions of the receptors and to substantial differences in their interactions with co-regulators and other nuclear proteins [61, 62]. In cell culture GR-MR heterodimerisation has been demonstrated, and the heterodimer exerted a greater effect upon transcription than either homodimer [63, 64], however the relevance of this mechanism *in vivo* has not yet been proven.

Progesterone Receptor (PR)

Progesterone and related progestins regulate several processes mostly related to fertility in females. They are potent differentiation agents in uterine and breast tissues and increasing levels are associated with pregnancy. Two isoforms of the progesterone receptor, A and B, generated as splice variants, have been well characterised [65, 66]. PRA is truncated in the ligand-binding domain and is unresponsive to hormones. PRA can act as a potent repressor of ligand activated PRB transactivation, apparently by direct competition for the DNA response element binding site [66, 67]. The ratio of the two isoforms is regulated and their relative concentrations differ substantially in different tissues and different developmental stages [68-70].

1.2.3 Steroid Hormones

Steroid Hormone Synthesis

Steroid hormone biosynthesis (Figure 1-4) occurs primarily in the adrenal cortex, gonads, and placenta. Cholesterol is taken up from the serum lipoproteins and enters into a pathway whose early intermediates are common for all the hormones. Progesterone is the earliest steroid hormone product of this pathway and acts as an intermediate that is processed further in all tissues except the ovary. After ovulation, progesterone is secreted from the ovary into the serum by granulosa cells rather than being converted to estrogens by the thecal cells. Progesterone is processed to mineralocorticoids, androgens or glucocorticoids in the adrenal, or to testosterone by the testes. Steroid hormones are made by a series of biosynthetic enzymes and are metabolised by enzymes known as mixed function oxidases (MFOs) that are part of the cytochrome P450 (CYP) superfamily of enzymes. CYP enzymes are also involved and in the detoxification of xenobiotics. The catabolism of steroid hormones involves sequential reduction and hydroxylation yielding conjugated steroids ready for elimination, which is mostly through the urine. This steroid catabolism takes place primarily in the liver.

Steroid hormone levels are regulated under the control of the hypothalamic-pituitary axis. Various sensors are in continuous communication with the hypothalamus, which is located just behind the anterior section of the brain. These signals regulate the release of a number of factors from specialised hypothalamic neurons. The hypothalamus is physically connected to the pituitary gland by specialised capillary networks, which enable rapid hormonal communication between the two. In response to stimulation from the hypothalamus, the pituitary releases hormones into the circulatory system. These gonadotropins regulate steroid production and metabolism. Most steroid hormones also act upon the hypothalamus, the pituitary, or both to regulate the production and or secretion of the hormones responsible for stimulating their own synthesis and release. Such feedback loops are common features of many hormonal pathways.

Estrogens

Estrogens (estradiol, estrone, estriol) are derived from specific androgens via aromatisation of the A-ring. They are found at very low levels in the serum compared to most other steroid hormones, with estradiol varying between 0.06 and 0.6 ng/mL in females through the estrous cycle. During pregnancy the primary source of estrogen synthesis shifts to the placenta, from where estrone and estradiol are produced from DHEA-S from both the fetal and maternal adrenals and estriol is synthesised from precursors originating on the fetal side. Estrogens are important in the development and function of the brain, skin and female reproductive tissues and breasts. In addition, they play many roles in general health and development that are not gender specific such as the regulation of serum lipoproteins, calcium levels and bone density.

Estrogens have also been shown be important in male development and fertility, including in masculinisation of the brain [71], and sperm maturation in the epididymis [72, 73]. The regulation of estrogen synthesis is complex, particularly in females where levels are continually cycling. Ovarian production is principally controlled by two pituitary hormones, luteinising hormone (LH) and follicle stimulating hormone (FSH), which stimulate increased serum estrogen. As potent stimulators of breast tissue growth, estrogens are closely associated with breast cancer, and several chemotherapeutics are ER antagonists.

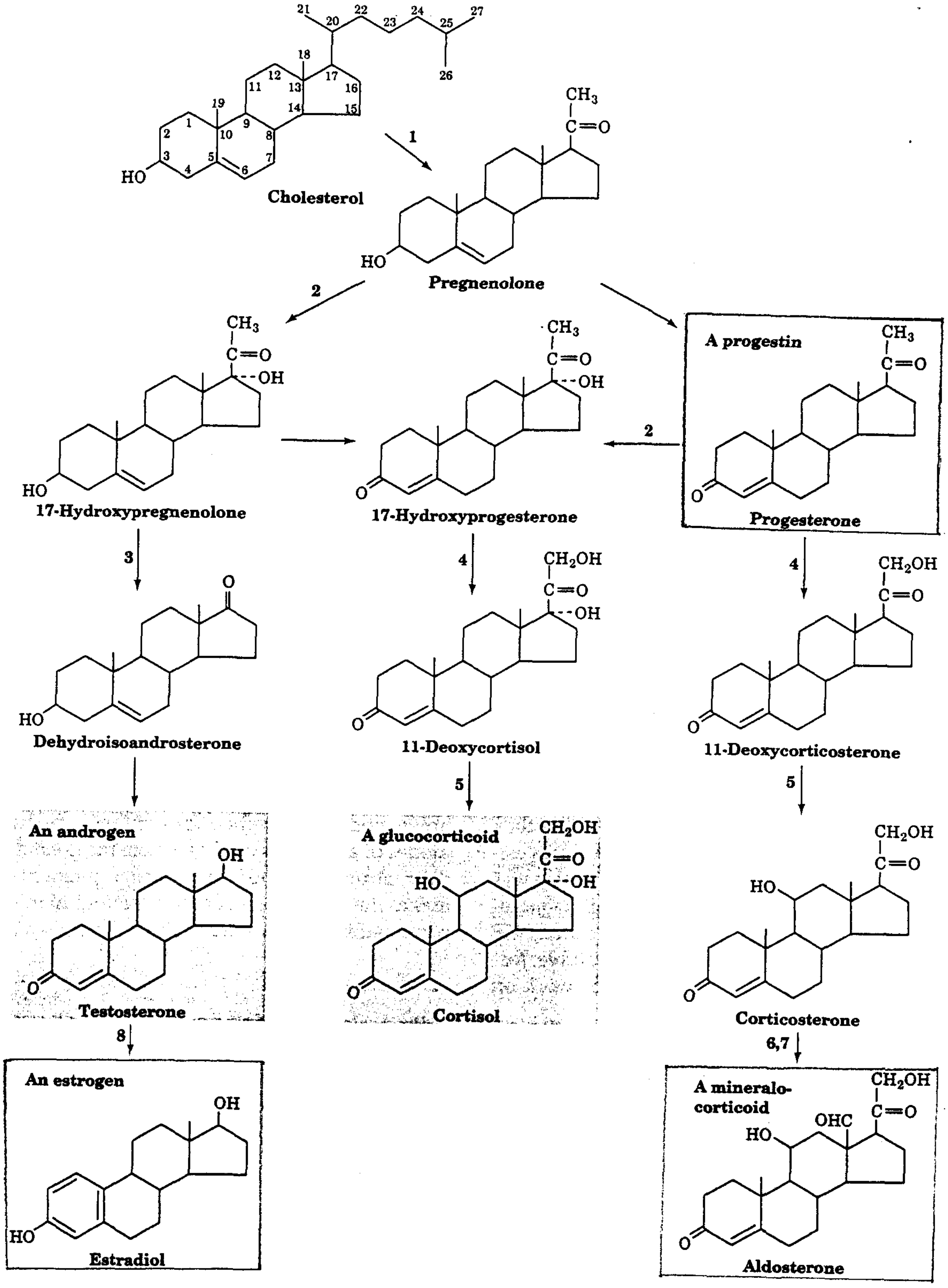


Figure 1-4: Synthesis and structure of steroid hormones.

Shaded structures represent the archetypal member of each the five steroid hormone classes. From Voet and Voet, [74].

Progesterone

As outlined above progesterone is a synthetic precursor of the other steroid hormones. Synthesis occurs primarily in the corpus luteum after ovulation to prepare the female for a possible pregnancy. If implantation of a fertilised ovum occurs, progesterone production shifts to the placenta. Although largely viewed as a modulator of estrogen action during the menstrual cycle and pregnancy, there is increasing evidence for important roles for progesterone beyond this context.

Glucocorticoids

Glucocorticoids, primarily cortisol, are produced by the adrenal glands and represent the most abundant of the steroid hormones in the serum. In humans cortisol levels fluctuate between 50 and 160 ng/mL through the course of a regular daily cycle, being highest after waking. Glucocorticoid production in the adrenal is stimulated by adreno-corticotrophic hormone (ACTH), secreted by the pituitary gland. ACTH release is, in turn, stimulated by corticotrophin-releasing hormone (CRH) from the hypothalamus, and both cortisol and ACTH act as negative feedback regulators upon the hypothalamus, inhibiting the release of CRH. Numerous stimuli can promote CRH production and release.

Glucocorticoid receptors are found in almost every cell and the effects of cortisol are correspondingly diverse. Some of the best-characterised roles of cortisol are in regulation of the immune system, and in carbohydrate metabolism. Suppression of the inflammatory response by glucocorticoids is achieved on several fronts. Increased T-cell apoptosis and transrepression of genes encoding proinflammatory factors are the major mechanisms [75-77]. The GR has been demonstrated to interact directly with other transcription factors, particularly AP-1 and NF-κβ, thereby inhibiting their ability to regulate the expression of their target genes [78]. In addition, the gene encoding a subunit of Iκβ, a partner protein of NF-κβ that sequesters it to the cytosol, has a GRE in its promoter region [79, 80]. The major effects of cortisol on glucose homeostasis are two-fold. Cortisol decreases glucose uptake by many cells, especially the liver, thus helping to maintain serum glucose levels. In the liver, cortisol stimulates gluconeogenesis from fatty acids and proteins and inhibits pathways that utilise glucose or remove it for storage. To further facilitate this, lipid mobilisation from adipose tissue is increased to supply the liver with substrate for gluconeogenesis.

Mineralocorticoids

Produced almost exclusively by the adrenal zona glomerulosa, the most potent mineralocorticoid is aldosterone although cortisol is also able to bind and activate the MR. In the normal adult human, levels range between 5 and 20 ng/ml although they can be elevated ten-fold under conditions of salt restriction. Production commences during fetal development and continues throughout life. Synthesis and release of aldosterone are regulated by ACTH from the pituitary gland. ACTH is released in response to internal and external stimuli collated through the hypothalamus. Angiotensin has also been shown to be a stimulant for increased aldosterone production. Mineralocorticoids act primarily upon the kidney to regulate electrolyte balance by stimulating sodium and chloride retention and excretion of potassium and hydrogen ions. Other effects of mineralocorticoids on regulating blood pressure are more direct and include modifying vascular tone.

Androgens

In addition to their function in gender determination and in development of male sexual characteristics, androgens (testosterone, dihydrotestosterone [DHT], dehydroepiandrosterone [DHEA], androstenedione) play an important role in many functions including tissue regeneration, especially the skin, bones, and muscles and in functioning of the female reproductive tract. Androstenedione and DHEA are generated by the adrenal gland and are present at similar levels in both male and female whereas testosterone is produced primarily in the reproductive tract (testes in males and ovaries of females). Serum testosterone levels differ substantially between the sexes (up to 10 ng/ml in adult males and 0.5 ng/ml in females).

During fetal development the adrenal gland acts as the primary source of androgens, producing DHEA-S, which is responsible for triggering sexual differentiation. At parturition this activity subsides and androgen levels remain low until adrenarche when testicular androgen synthesis increases and stimulates further development of male characteristics. In birds and some mammalian species (e.g. rodents), there is evidence of a testosterone spike around the time of parturition. This appears crucial to the masculinisation of the neural system because if it is blocked or absent affected individuals fail to acquire male mating behaviours post puberty and normally do not reproduce [81-83].

Most of the testosterone from the testis goes into peripheral circulation and is bound to serum binding proteins (androgen binding protein and steroid hormone binding globulin), with 1 to 2% of the total remaining unbound in the plasma as free testosterone. A fraction of the testosterone diffuses from the Leydig cells into the seminiferous tubules, Sertoli cells and germ cells for the maintenance of spermatogenesis and to the genital tract, including the epididymis. Testosterone is converted to dihydrotestosterone (DHT) by 5α-reductase within the prostate and several other target tissues. DHT is a more potent androgen than testosterone because it has a ten-fold lower dissociation constant for AR (approximately 0.1 nM versus 1 nM respectively).

Thyroid Hormones

The hypothalamic-pituitary axis also regulates the levels of hormone secretion by the thyroid gland. Thyroid hormones thyroxine (T4) and 3,5,3'-triiodothyronine (T3) are derived from tyrosine (Fig 1-5) and their secretion is stimulated by thyroid stimulating hormone (TSH) from the pituitary. Thyroid hormones share many similarities with the steroid hormones. In the plasma they are sequestered by thyroid binding proteins that actually retain over 99% of the total serum T4 and T3. The free hormone fraction can cross the plasma membrane of target cells and binds to an intracellular receptor that is a member of the nuclear receptor superfamily. Thyroid hormones regulate an array of important cellular processes through development and into adult life. They are particularly well characterised for their role in amphibian metamorphosis, but they play many diverse and critical roles in humans including maturation of the brain, central nervous system and lung and regulation of growth through transactivation of the gene for growth hormone, as well as in oxygen utilisation and regulation of energy metabolism.

The potent effects of steroid hormones in development and homeostasis combined with their relatively simple chemical structure have made steroid pathways popular targets for therapeutics. Many pharmaceuticals have been developed that influence steroid synthesis, release, or action either by inhibiting endogenous pathways or mimicking a component of it. Interestingly many of the successful steroid mimics that have been synthesised are structurally non-steroidal (Fig 1-6).

1.2.4 Mechanism of Action

When a steroid hormone enters into the target cell it binds to a nuclear hormone receptor stimulating a conformational shift in the receptor structure. This activated hormone-receptor complex can bind to specific DNA regulatory sequences and activate or repress specific genes. For effective endocrine regulation it is of utmost importance that the levels of individual hormones are tightly controlled in a temporal fashion to elicit the appropriate response in the desired time frame [84].

A steroid hormone receptor is not only able to bind its cognate hormone, but also other quite distinct molecules, albeit with different binding affinities [85-88]. Any molecule that can bind in the ligand binding pocket of a specific receptor is called a ligand. A hormone represents a physiological type of ligand that elicits the appropriate cellular response upon binding to the given receptor. Other ligands may bind the receptor, and their interaction may result in a high level (agonist) or a low level (partial agonist) of expression or may even block gene expression completely (antagonist).

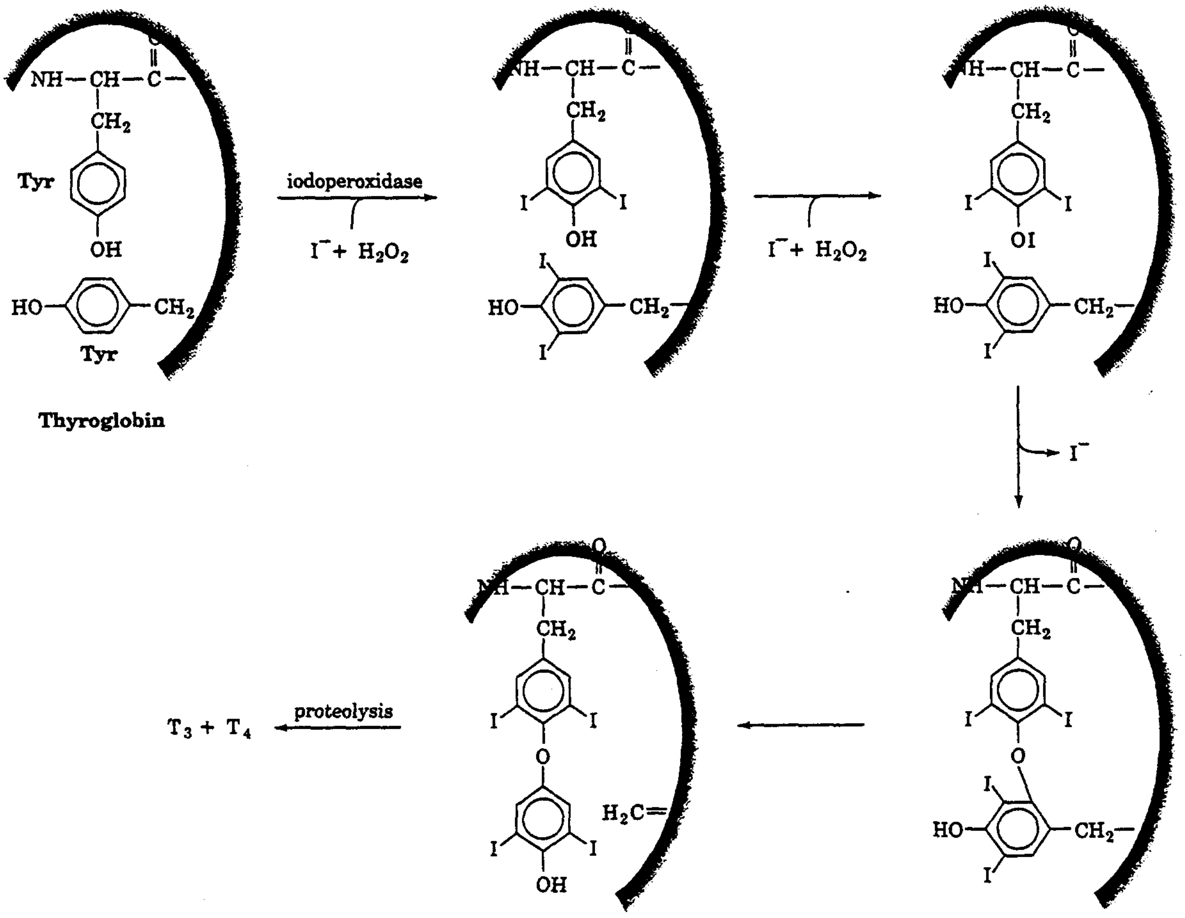
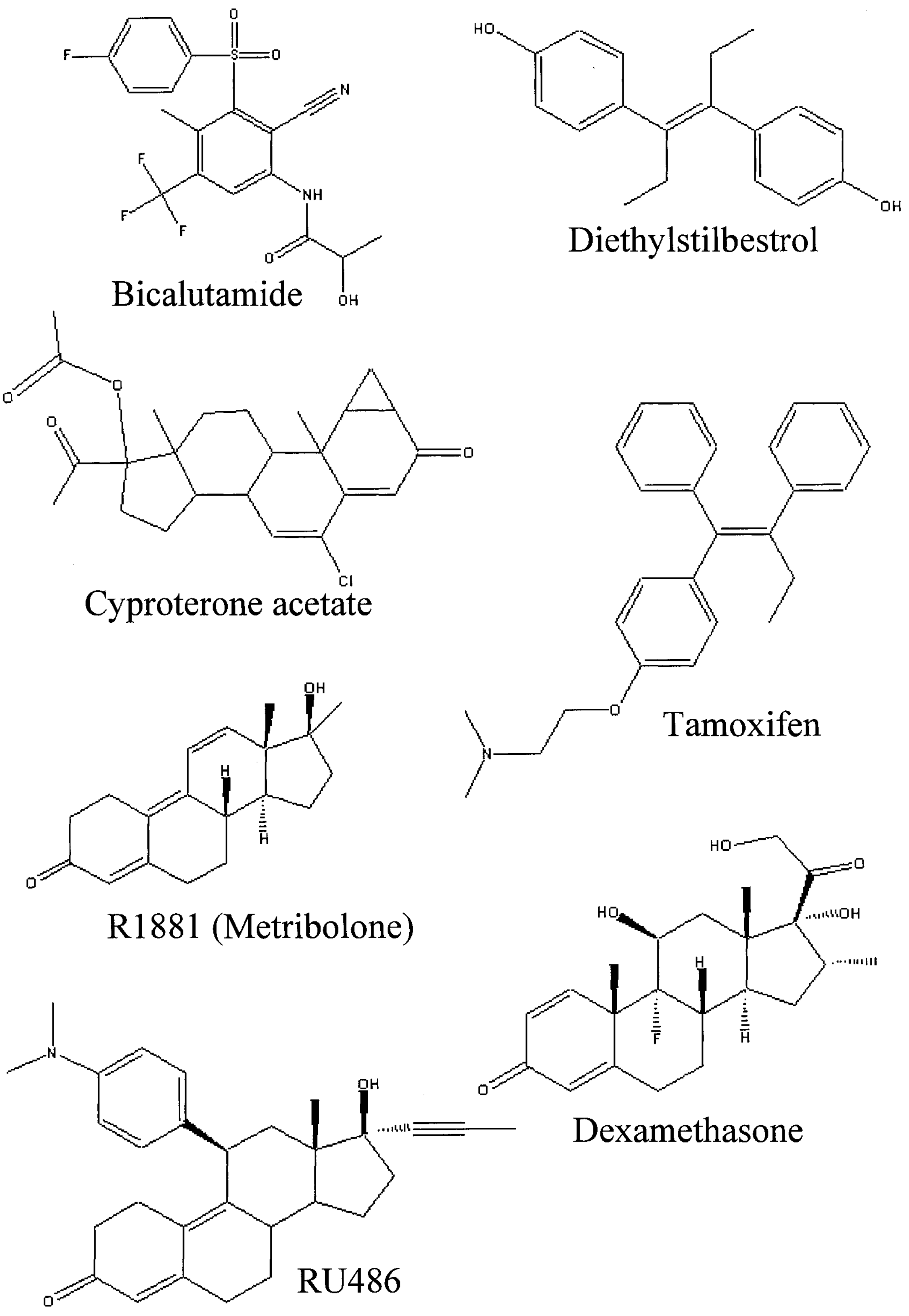


Figure 1-5: Synthesis and structure of thyroid hormones.
The biosynthesis of T3 and T4 in the thyroid gland via the iodination, rearrangement, and hydrolysis of thyroglobulin tyrosine residues. From Voet and Voet [74].



Bicalutamide

Diethylstilbestrol

Cyproterone acetate

Tamoxifen

R1881 (Metribolone)

Dexamethasone

RU486

Figure 1-6: Structure of some synthetic steroids

1.3 Regulation of Steroid Hormone Action

1.3.1 Interactions with the Basal Transcriptional Machinery

Eukaryotic cells have three different RNA-polymerases (I, II, and III), each specific for a particular set of genes with characteristic promoter regions. Promoters are associated with two types of transcription factors: the basal transcription factors (BTFs), which interact with core promoter elements proximal to the gene's five-prime end, and the sequence specific transcription factors, of which the steroid receptors are one example. The steroid receptors interact with more distal elements, usually located several hundred bases or more upstream. The basal factors are fundamental for RNA polymerase recruitment and transcriptional initiation while the sequence specific factors perform a regulatory role enhancing or restricting transcription. Steroid receptor response elements have been almost exclusively identified in promoters for genes transcribed by RNA polymerase II (Pol II), particularly those used to generate mRNAs for the production of proteins.

Extensive *in vitro* experimentation has generated a model for the assembly of the BTFs on the core promoter elements [89]. Most Pol II genes have a TATA box sequence located about 25 bp upstream of the transcription start site. The first step in the assembly of the functional Pol II complex appears to be the binding to this sequence of $TF_{II}D$, which comprises the TATA box binding protein (TBP) and associated factors ($TAF_{II}s$). Subsequent recruitment of $TF_{II}B$, $TF_{II}F$ and Pol II itself yields a minimal functional complex *in vitro*. Several other BTFs have been identified and it seems that they are also normally present in the complete transcription complex *in vivo*. The addition on $TF_{II}A$, $TF_{II}E$ and $TF_{II}H$ *in vitro* significantly increases the rate of transcription and stabilises the protein complex on the DNA. Furthermore, it seems that it is in part through these proteins that many of the distally-located transcriptional modulators are able to exert an effect upon the transcription rate.

The steroid receptors have been shown to interact with several of the $TAF_{II}s$ that form part of $TF_{II}D$. *In vitro* experiments have shown that human $TAF_{II}30$ has specific interactions with the transactivation region of the estrogen receptor hormone binding domain [90]. Glucocorticoid receptor has also been shown to have direct interactions with the $TF_{II}D$ complex, which are important mediators of trans-activation by glucocorticoids

[91]. Meanwhile, AR has been shown to interact directly with several regions of $TF_{II}F$ [92].

The major effects of steroid receptors upon the BTFs are achieved by indirect means. Co-regulators such as those discussed below are able to interact both with the steroid receptors and simultaneously with the BTFs and thereby facilitate the assembly of the transcription complex at the start site. In some cases the interaction is with another transcription factors that in turn regulates transcription.

It has been shown that under distinct physiological conditions the presence of hormone response elements can synergistically regulate the effects of other transcription factors [93]. Optimal induction by GR or PR of genes regulated by the mouse mammary tumour virus promoter has been demonstrated to require the transcription factors NF-1 and Oct-1, and GR and PR have been shown to be able to interact directly with Oct-1 [94]. Similar interactions on a number of other promoters have also been reported with both positive and negative regulatory outcomes [79, 95-97].

1.3.2 Co-Regulatory Proteins

An array of proteins has been isolated based upon the ability to bind to the steroid receptors, and these proteins have been sequenced and characterised. Once a co-regulator for one receptor is identified, its ability to modulate the transcriptional activity of other receptors is assessed. Each steroid receptor interacts with several coregulatory proteins to form a large complex on the DNA, which in turn interacts with various components of the basal transcriptional machinery and with the DNA directly to regulate transcription. Some of the co-regulatory components appear to be almost ubiquitous and are essential to effective regulation of gene expression by the receptors. Others seem to be tissue- or cell type-specific factors, allowing for fine-tuning or specialised responsiveness of gene expression in that cell. Similarly, some factors are able to interact with all the steroid hormone receptors and in some cases many other transcription factors as well, while others appear to form specific interactions only with one receptor type. Some of the properties of the more ubiquitous factors are outlined below.

Steroid Receptor Coactivator-1

In 1995 a protein was identified that interacted with the human progesterone receptor AF-2 domain and reversed the squelching effect of ER when co-expressed in cell culture [98]. This protein was named steroid receptor coactivator-1 (SRC-1) and it was suggested that this protein was essential for all steroid receptors to achieve full transcriptional activation. This protein has subsequently been shown to interact directly with the estrogen and glucocorticoid receptors [99] and to be the first of a family of related proteins that share significant homology with carboxyl-terminal portions of the 156 kDa N-CoA protein isolated from mice [99, 100]. Innate acetyltransferase activity has been characterised in SRC-1, that is linked to its ability to activate transcription [101]. This enzymatic activity enabled the acetylation of histones H3 and H4. SRC-1 and related proteins have been shown to bind to TBP and to TFIIB in the basal transcriptional complex [102].

Co-regulatory proteins	**Related proteins**	**Known receptor**	**Further information**
SRC-1	P160, N-CoA, GRIP1, TIF2, AIB1, p/CIP	ER, PR, GR, AR	A histone acetyltransferase and a component of N-CoA
RIP140	p140	ER	
TIF1		ER, PR	Is related to T_{18} oncogene 26S
TRIP1	SUG1	ER	A proteasome subunit in yeast
ARA70	RFG	AR	
P/CAF	GCN5		no interactions
CBP/p300		AR, ER, GR, PR	A histone acetyltransferase and co- integrator of multiple signals
N-CoR		ER, GR, PR	
SMRT		ER, GR	
HDAC		no interactions	

Table 1-1: Some co-regulatory proteins of steroid hormone receptors.
The second column gives the names of homologues. Column three indicates which of the steroid receptors they have been shown to interact with and the fourth column summarises what information about the protein is currently available to help elucidate the mechanism by which they are able to promote gene transcription.

CBP/p300 Co-integrator

CBP (cAMP response element binding protein binding protein) and p300 (adenovirus E1A associated protein) have been characterised as ubiquitous co-activators that are able to interact with almost all members of the nuclear receptor superfamily as well as several other transcription factors through regions in the amino-terminal portion of the protein [103-105]. This multifunctional potential has led to the labelling of these proteins as co-integrators since it appears they are able to help the cell coordinate a response to several stimuli [99, 106, 107]. Furthermore, it has been shown that CBP interacts with N-CoA and members of the SRC-1 family, as well as an RNA helicase A of the Pol II complex and other proteins of the basal transcriptional machinery through regions in the carboxyl-terminal portion of the protein [108, 109]. In addition to the multiple domains of the protein that interact with the various transcription factors, CBP contains acetyltransferase activity [110]. It appears that CBP acts co-operatively with SRC-1 to activate steroid hormone mediated transcription [111].

P300/CBP Associated Factor (P/CAF)

A factor, P/CAF, was identified which competes with adenoviral early region 1A (E1A) for binding to p300 and was also able to bind CBP [112, 113]. This protein is homologous to the yeast transcriptional activator GCN5 both in its sequence and in sharing histone acetylation activity. The same study also identified an additional human GCN5 homologue encoded by a different gene with a variant tissue expression pattern from P/CAF. Unlike CBP/p300 and SRC-1 type factors, there is no evidence to indicate that P/CAF or hGCN5 can interact directly with the steroid hormone receptors or with other components of the basal transcriptional machinery.

For many of these factors the interaction with the steroid receptors appears to be through the AF-2 domain in the E-region (Figure 1-3A) of the steroid receptor. A repeated conserved sequence (LXXLL) found in many co-regulatory proteins is believed to form a part of the interface with the AF-2 region of steroid receptors, although other regions are also necessary [114]. The ability of these co-regulatory proteins to bind to the steroid receptor E-region has been repeatedly demonstrated to be dependent upon the presence of ligand.

The structural models for the AF-2 domain determined from the RAR/ RXR/ TR crystal structures discussed previously showed that the incorporation of ligand into the LBD leads to a re-alignment of the three helices (3, 4, and 12), which are all essential components of the AF-2 domain. Presumably this agonist-induced conformational shift is required to create the co-activator-binding interface. Furthermore, the published ER LBD structure [115] in complex with both the agonist, 17-β-estradiol and the antagonist raloxifen, seems to demonstrate that the differentiation between agonist and antagonist response by a receptor may be determined by the ligand-induced conformation in the ligand-binding domain. The position of helix 12 in particular was found to be significantly different in the antagonist bound receptor compared to the receptor bound by 17-β-estradiol.

1.3.3 Transcriptional Repression

Two ubiquitous proteins have been identified that appear to have key roles in negative gene regulation. These co-repressors were initially isolated because they bind directly to three members of the nuclear receptor superfamily, the thyroid, retinoic acid and the retinoid X receptors, in their unliganded repressor state [12].

Unlike the steroid receptors the retinoid/thyroid receptors are only found in the cell nucleus and can form homo- or heterodimeric complexes which bind to the DNA as monomers or dimers with or without ligand. Typically the retinoid / thyroid receptors behave as repressors of genes when bound to DNA in the absence of ligand, and are converted to transcriptional activators upon ligand binding. Although there is now a growing sense that steroid receptors can bind to DNA in an unliganded state, most of the evidence for gene repression by these receptors relies upon the use of man-made receptor antagonists and there is only scant evidence to support the idea that unliganded steroid receptors can bind to DNA *in vivo*. It has been shown that antagonist-bound steroid hormone receptors can associate with the same repressor complex as has been identified as mediating thyroid / retinoid transcriptional repression [116, 117]. The major conserved components of this complex are outlined below.

Nuclear Co-repressor

Nuclear corepressor (N-CoR) is a 270kDa protein first isolated from mouse cells because of its interaction with unliganded TRβ and since demonstrated to be widely expressed in many mammalian cell lines [117]. Regions within the protein's carboxyl-terminus interact with nuclear receptors through their hinge region and LBD. The regions essential for effective gene repression have been mapped to the protein's amino-terminus by truncation studies [116]. N-CoR was shown to be dissociated from the receptor by ligand-binding [118].

A closely related protein, the silencing mediator for retinoid and thyroid receptors (SMRT), is a 168 kDa protein in humans and has been shown to be almost ubiquitously expressed and to interact with the steroid receptors [119, 120]. SMRT is functionally homologous with the first 140 amino acids of N-CoR. Indeed the receptor interaction domains of the two proteins share nearly 50% sequence identity, suggesting a possible common evolutionary precursor [117].

Histone deacetylase (HDAC) activity was identified and has been shown to be present in cell nuclei in complexes containing SMRT or N-CoR [121]. It seems that the HDAC does not directly interact with the steroid receptor but that SMRT or N-CoR provide an attachment surface for recruitment of this enzyme to the trans-repressor complex. The over-expression of this protein in transient transfection assays has been shown to lead to a ten-fold repression of reporter gene expression and cell senescence [122-125]. As the name suggests, this protein is presumed to act by antagonising the histone acetylation activity associated with many transcriptional activators.

SWI/SNF Nucleosome Remodelling

The SWI/SNF complex in yeast, and its mammalian homologues, facilitate transcriptional activation by opposing chromatin-dependent transrepression. These multi-protein complexes, comprising nine to twelve subunits in mammals, are able to disrupt chromatin in an ATP dependent manner and it was initially believed that this activity precluded the binding of the sequence specific transcription factors to the DNA [126-128]. The steroid receptors can interact directly with several components of the SWI/SNF complexes and may activate them [126-128]. The steroid receptors are able to bind chromatin and recruit SWI/SNF complexes to the DNA [129, 130]. This chromatin binding is in contrast to other transcription factors that are only able to access their DNA binding sites subsequent to SWI/SNF mediated chromatin disruption [129, 131].

1.3.4 Effects of Other Signalling Pathways

It has been demonstrated that peptide hormones can influence the transcriptional regulation performed by steroid hormones. For example, both epidermal growth factor and insulin have been shown to be capable of increasing estrogen-stimulated gene activation [132, 133]. In some cases, such peptide hormones can activate steroid receptor-mediated gene transcription in the absence of the steroid hormone [134-136]. This activation appears to result from the phosphorylation of the steroid receptors by microtubule associated protein kinase or protein kinase A at specific serine and tyrosine residues [137-139]. This theory is further supported by the identification of several steroid response elements in the enhancer region of the p21 and p27 genes, two proteins that regulate the activity of cellular kinase enzymes [140-142].

1.3.5 Model for Steroid Hormone Action

In the nucleus, the structure of genes in chromosomes is more complex than a simple DNA polymer. The double stranded helix is wound around clusters of histone proteins to form nucleosomes and these can be further coiled to form a 30 nM fibre structure. This tertiary structural arrangement has been shown to be a potent regulator of gene transcriptional activation. Before the basal transcriptional machinery can be assembled on the promoter sequence for a gene, the DNA must be unwound from the nucleosomal structure [143, 144]. A two-step model has been developed to explain the mechanism by which steroid receptors are able to initiate gene transcription from such a site (Figure 1-7). Stage one involves the binding of receptors in complex with coregulators to the DNA. Subsequently, additional factors are recruited to the DNA containing a mixture of ubiquitous and specialised co-regulatory factors. In the second phase, this complex promotes the uncoiling of the promoter region through modification of the histone structure, weakening the interaction with the DNA and facilitating assembly and activity of the basal transcriptional machinery. Gene repression can occur when, due to the conformation of the receptor ligand-binding domain (antagonist bound

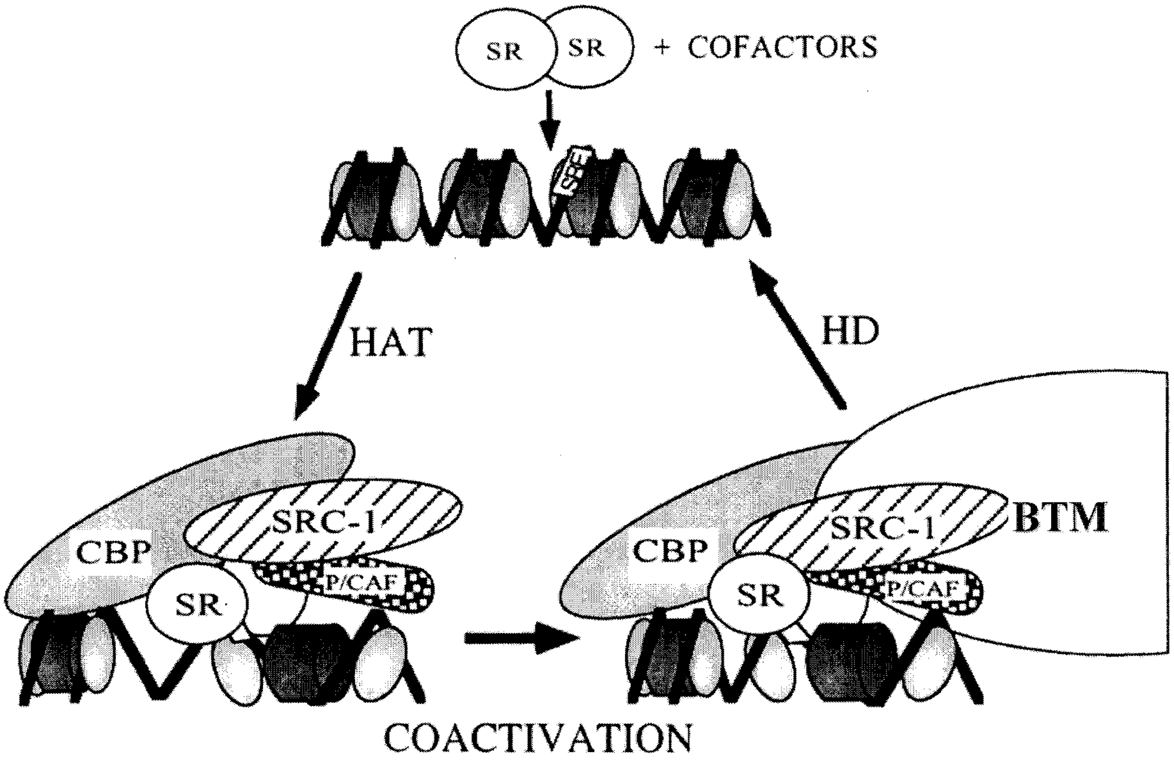


Figure 1-7: Model for steroid hormone transcriptional activating complex. SR = steroid receptor, SRC-1 = steroid receptor coactivator-1, CBP = cAMP response element binding protein, HAT = histone acetyltransferase, HD = Histone deacetylase, P/CAF = p300/CBP associated factor, BTM = basal transcriptional machinery.
Adapted from Jenster *et al.*, [165]

or perhaps unliganded), a co-repressor complex is recruited.

This theory has been supported by the discovery that, unlike the basal transcriptional machinery, thyroid hormone receptors are able to bind to nucleosomal structures, even when portions of the DNA are physically coiled around the histone proteins. In these cases, the crucial residues of the response element sequence are surface exposed in the nucleosomal structure. This pattern appears to be repeated in steroid receptors [146]. Alternatively, it has been shown that the DNA response elements are located in the linker sequences, which connect adjacent nucleosomes making them more available to the receptor dimer [147, 148].

Once the steroid receptors are attached to the DNA they recruit and apparently activate co-regulatory proteins either singly or as a pre-formed complex. Co-regulators are thought to bind to residues in helixes 3,4 and 12 in the LBD, which form the core of the AF-2 domain of the steroid receptors. There is evidence for a cooperative interaction between this and the AF-1 domain in the carboxyl terminal portion of the protein [10].

The exact mechanism by which an activator complex opens up the essential promoter elements for BTFs binding remains somewhat controversial. Many researchers have proposed that the core histone proteins are acetylated and this decreases their affinity for the DNA (presumably by reducing their overall positive charge), thus promoting nucleosome disassembly [131]. This theory has gained substantial support since many of the co-activating proteins have been shown to have inherent acetyltransferase activity and are indeed able to acetylate some or all of the histone proteins. Furthermore, it has been demonstrated that co-inhibitory complexes incorporate enzymes with histone deacetylase activity [131].

There is evidence indicating that several of the steroid receptors themselves and other transcriptional co-factors can be acetylated by the proteins in the coactivator complexes [149-153]. It is clear that there are further interactions between the steroid receptors, co-regulators and the BTF elements that may affect the stability of the complex on the DNA and generally affect the potential to initiate transcription.

1.3.6 Steroid Receptors and the Aryl Hydrocarbon Receptor

Polychlorinated dibenzodioxins (PCDDs) and some other aromatic hydrocarbons bind to the aryl hydrocarbon receptor (AhR), which is a member of the βHLH PAS domain family of ligand-responsive transcription factors from the steroid receptors [154-156]. Activated AhR regulates the transcription of Phase I enzymes in the metabolism of lipophilic substrates and simultaneously activates the Phase II enzymes, which are conjugating enzymes such as glutathione s-transferase (GST) [157]. After toxins have been modified by the Phase I enzymes they can be conjugated by Phase II enzymes to increase solubility and facilitate excretion from the body. As with the steroid receptors, a number of structurally distinct ligands can interact with the AhR each producing different biological effects. For instance, PCDDs primarily activate CYP1A1 and elicit deleterious effects, while some compounds such as Oltipraz activate the Phase II pathway to a higher degree and appear to be protective for cancer [158].

It has been demonstrated that AhR is able to influence the response to steroid hormone stimulus and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) has been characterised as a potent anti-estrogen. These effects can be produced at a number of levels: effects upon metabolic enzymes may lead to increased elimination of steroid hormones by the liver [159]; competition for co-regulatory components within common target cells may lead to squelching of the steroid response; and in some cases there are negative dioxin response elements in the regulatory regions of genes whose transcription is increased by steroid hormone action [160].

1.4 Environmental Hormones

1.4.1 Phytohormones

Many plant-derived compounds have been identified that are able to produce steroid hormone-like effects. Almost exclusively these non-steroidal substances produce effects that are estrogenic or anti-estrogenic in nature. Increasingly these phytoestrogens are used as medicines in place of pharmaceutical estrogens by individuals persuaded that these products derived from natural sources are less harmful than conventional therapies. The evidence from scientific analysis of many of these remedies is inconclusive to date but there is no doubt that several plant derived steroid products have some efficacy and may prove to be valuable additions to the pharmacopoeia.

1.4.2 Endocrine Disruption

About 63,000 chemicals are in common use worldwide, and up to 1000 new synthetic chemicals are marketed each year [161]. While these receive routine toxicity screening, direct endocrine activity is not specifically assessed. Concurrently, there has been a steady global increase in cancers of endocrine regulated tissues. For example, in Canada between 1982-1989 there was an annual increase in breast cancer incidence of 1.5%, in prostate cancer of 3.1%, and 2.4% in cancer of the testes [162]. While some increase can be attributed to improved detection, there has been speculation that increases in endocrine sensitive cancers, in addition to other conditions such as birth defects, endometriosis, and immune disorders, may be attributed in part to the effect of environmental contaminants on the different components of the endocrine system [163-165].

These contaminants include organochlorines such as pesticides as well as hydrocarbons such as alkyl phenols. Compounds from both of these classes have been demonstrated to be hormonal mimics that bind to the estrogen receptor (ER) and interfere with normal estrogenic functions [88, 164, 166]. Interaction with other hormone receptors, such as the androgen receptor (AR), progesterone receptor (PR), and glucocorticoid receptor (GR) have largely not been addressed, but are suspected because these receptors also are able to bind to molecules that are structurally distinct from their cognate physiological steroidal ligands.

Numerous synthetic therapeutic steroid agonists and antagonists have been developed for use in the regulation of fertility, immunosuppression, and birth control and for the treatment of cancer. The structural variety of these compounds has demonstrated that the ligand-binding pocket of steroid receptors has an unexpected amount of latitude in ligand-binding specificity [85, 87]. Recently, this ligand mimicry has been observed with substances from environmental sources, such as nonylphenol (NP) and DDT and other pesticides, which bind to the ER and interfere with the natural action of estrogen [38, 86-88, 167]. The structural diversity of these receptor-binding molecules makes it impossible to predict biological effect from structural analysis alone. Thus, biological assays are required to directly investigate the functional consequences of the binding of a potential ligand with a receptor.

Endocrine disruption of steroid function can occur by a less direct means mediated by AhR. PCDDs have been shown to have profound effects on cancer, fertility, and immuno-responsiveness [157, 168-170]. Investigations with ER have shown that the PCDDs acting through AhR interfere with estrogenic responses. On some estrogen responsive promoters the mechanism may be one of AhR competition with ER for DNA binding targets [160, 171]. However, other studies have implicated a mechanism that occurs without direct DNA binding competition [172-175]. Collectively, a number of studies suggest that the latter mechanism may be one of sequestering a limiting co-activator protein, which can competitively bind to both AhR and ER in a mutually exclusive manner [176]. Since these co-regulators, such as CBP and the SRC family interact with most steroid receptors, it suggests that PCDD-liganded AhR may also indirectly interfere with the functions of androgens, glucocorticoids and progestins.

Endocrine Disruption and Birth Defects

Hypospadias occur in one in every 125 live male births in North America. This incidence represents a doubling from the levels reported in 1968 [177]. Such profound increases have also been reported across Western Europe but were less marked in many developing countries [177].

Two incidents in Asian countries have highlighted the potential negative impact on children born to women exposed to endocrine-disrupting substances while pregnant. In both cases contamination of rice oil with PCBs, furans and PCDDs occurred within the manufacturing facility but was not detected until after the product was on the market. Health effects were observed in both the exposed individuals and in their offspring born after the exposure. In exposed adults there were acute toxic effects, primarily chloracne, and over long term monitoring a significant increase in primary liver cancer has been reported [178]. Children born to mothers who consumed the contaminated product were followed and a number of developmental abnormalities were observed, including lower body weight and height, higher activity levels, greater incidence of behaviour problems, and lower IQ scores [179, 180]. Similar neurological impairment has been reported in North America for populations consuming fish from the Great Lakes [181, 182].

Endocrine Disruption and Cancer

In addition to the increased cancer rates amongst populations exposed to putative endocrine-disrupting compounds through contaminated rice oil as described above other cancers have been proposed as potentially sensitive due to their known endocrine aetiology. The association of PCBs and DDT and an increase in breast cancer rate has been studied but remains speculative ([183-186]. Early studies suggested a strong correlation between plasma DDT and DDE concentration and breast cancer incidence but in larger case-control follow-up studies no significant correlation has been consistently reported [187, 188]. Several epidemiological studies have indicated a link between exposure to certain organochlorine pesticides and prostate cancer incidence, although others have found no significant association [189-192].

DES Exposure

Female fetuses exposed by maternal intake of diethylstilbestrol (DES) have shown a significant increase in vaginal and cervical cancers [193]. In addition, prenatal exposure to DES appears to sensitise certain endocrine responsive tissues to estrogens in later life, which has been hypothesized to contribute to an increased risk of breast, uterine, and prostate cancers [194]. DES has been shown to bind poorly to serum hormone binding proteins that normally sequester the majority of the steroids in circulation. The resultant relative increase in the concentration of this non-steroidal estrogen able to move freely across the plasma membrane may account for some of its potency *in vivo*. This same phenomenon may well apply to other non-steroidal hormone mimics.

1.4.3 Androgen Disrupting Chemicals

Although attention in the field of endocrine disruption has focussed primarily upon compounds capable of interfering with estrogen pathways, several compounds capable of interfering with androgen-mediated pathways have also been identified. These include metabolites of the fungicide vinclozolin [195-197], *p,p'*-DDE, a major metabolite of DDT [198, 199], PCBs [200], bisphenol A, butyl benzyl pthalate [201], kepone [202] and nonylphenol [203].

1.4.4 Hormonal Levels Through Life

Fetal development is a highly sensitive time for endocrine disruption as cells are initiated into their differentiation pathways. However, increased exposure to hormones in adulthood has also been implicated in an increased risk for cancers of the breast, endometrium, and prostate [204-206]. In the case of breast cancer, the length of time between menarche and menopause as well as adipose levels have been correlated to a positive increase in breast cancer [207, 208]. These factors in women prolong or elevate estradiol levels, which results in increased proliferation of breast epithelial cells and simultaneously appears to suppress the tumour suppressor, retinoblastoma susceptibility gene product [209]. Interestingly, androgens may increase the risk for breast cancer even more so than estrogens [210]. In pre-menopausal women, high serum testosterone or DHT levels imply up to 3.4-fold increased risk [211], whereas in postmenopausal women high levels of testosterone and androstenediol in the urine are linked to a 4.7-fold increased risk [207]. Likewise, a strong positive correlation with endometrial cancer was reported for obese women with high levels of estrogen and/or androstenedione in their urine, or women on unopposed estrogen replacement therapy [208, 212]. In the case of prostate cancer there has been speculation that higher levels of circulating testosterone may increase the risk of developing this cancer [204, 205, 213, 214].

1.4.5 Immunosuppression

Chronic immunosuppression has been shown to lead to an increased risk of many cancers, including non-Hodgkin's lymphoma, skin cancer, and cancers of the urinary tract [215-217]. Carcinomas in marine mammals have also been attributed to the immunosuppressive effects of the heavy load of PCBs and organochlorines found in these animals at the top of the food chain [218]. Populations of seals in the North Sea were severely impacted by a distemper outbreak in the mid 1990s, the rapid spread of which was attributed to the weakening of the seals' immune systems as a result of their accumulated body burdens of organochlorines [219]. The immune system can be disrupted either with chemicals that interact with the AhR, or with GR, PR, or ER [220, 221]. This may in part result from cross talk between steroid receptors and AhR, which can interact with steroid hormone mediated cellular responses.

1.5 The Prostate

The prostate is a male sexual accessory organ that is involved in seminal fluid production. It is located surrounding the urethra at the base of the bladder. In rodents it consists of three regions, the ventral, dorsal and lateral, which roughly correspond to the peripheral, central and transition zones respectively of the human prostate. The ventral lobe comprises the majority of the normal prostatic volume and its histological appearance is characterised by small, acinar spaces lined by tall, columnar, secretory epithelial cells. Prostatic acini are embedded in smooth muscle stroma whose function may be to enhance the emptying of prostatic secretions into the urethra at the time of ejaculation.

1.5.1 Androgen Regulation of Prostate Development

The prostate is highly responsive to steroid hormone levels in terms of its differentiation, development, and growth [222-225]. Differentiation of the prostate tissue during fetal development is dependent on androgen function. At puberty the human prostate grows exponentially from one gram in the child to 20 grams at adulthood in response to rising testicular androgens. Castration prior to puberty prevents the prostate from developing even if testosterone is re-administered in later life. In rodents the post-partum testosterone spike seems to be critical for programming the adult structure and function of the prostate gland [226]. Administration of anti-androgens to an adult male causes the prostate to involute to the pre-pubescent size due to apoptotic regression [227, 228] illustrating that the continued presence of androgen is required for homeostasis of the tissue.

1.5.2 Prostate Cancer

Prostate cancer is currently the most commonly diagnosed cancer and the second highest cause of cancer death in North American men. The lifetime risk of a prostate cancer diagnosis for a Canadian male has increased over the past 20 years, which is in part due to the introduction of screening men over the age of 45 for increasing prostate-specific antigen (PSA-tumour marker). Prostate cancer incidence and mortality increases markedly with age, but despite this increased risk and intensive research efforts, the molecular mechanisms associated with the progression of prostate cancer have yet to be elucidated. Several studies have recently reported a positive correlation between pesticide exposure and prostate cancer amongst farmers and pesticide applicators [189-191, 229-232]. In addition it has been shown that workers exposed to high doses of PCBs had increased risk of prostate cancer [233]and two studies have correlated exposure to poly aromatic hydrocarbons (PAH), which act through the AhR, to increased prostate cancer risk [234, 235].

1.6 Rationale

Extensive study has demonstrated that a host of environmental contaminants are able to interfere with the normal function of the estrogen receptor *in vivo* and in a battery of *in vitro* assay systems. Limited evidence has been collected identifying a number of compounds that interact with the androgen signalling and bind to the androgen receptor. My hypothesis was that selected environmental contaminants are able to interact with androgen, glucocorticoid or estrogen receptors impacting receptor mediated gene transactivation *in vitro*. In addition I propose that those contaminants able to cause changes in androgen regulated gene expression *in vitro* can alter development of androgen-regulated endpoints *in vivo*.

To accomplish this a number of objectives were set.

Objective 1: The development a series of cell culture assays using the androgen, glucocorticoid or estrogen receptors in combination with the luciferase reporter gene under the control of receptor specific promoters in responsive human cell lines.

Objective 2: To utilise these assays to investigate the ability of hexachlorobenzene, β-endosulfan, DDT and related metabolites, pulp and paper mill by-products, and Fraser River sediment extracts to interfere with androgen, glucocorticoid or estrogen receptor mediated gene transactivation.

Objective 3: To investigate the ability of nonylphenol, octylphenol and nonylphenol ethoxylates to interfere with androgen, glucocorticoid or estrogen receptor mediated gene transactivation and ligand binding, and to investigate the effect of nonylphenol administration in mice.

Objective 4: To investigate the ability of Aroclor mixtures to interfere with androgen, glucocorticoid or estrogen receptor mediated gene transactivation and ligand binding, and to investigate the effect of Aroclor 1254 administration in mice.

Objective 5: To determine the effects of eight PCB congeners on androgen, glucocorticoid or estrogen receptor mediated gene transactivation and ligand binding, and to investigate the effect of 2,2',3,4'-tetrachlorobiphenyl (PCB 42) administration in mice.

Chapter II - Materials and Methods

2.1 Materials

2.1.1 Chemicals

PCBs were produced for industrial purposes and sold commercially as mixtures under a number of names, including Aroclor. These mixtures are composed of up to 209 congeners, although in each Aroclor mixture only a hundred or so congeners are present at detectable levels. Two Aroclors, 1254 (average mol wt.=328) and 1260 (average mol wt.=375.7), were tested in this study. These four digit identifiers indicate that the parent molecule has a 12 carbon backbone (12xx) and the percent chlorine content by weight of the mixture from the last two digits [236].

The Aroclors (1254 and 1260) and individual PCB congeners used in this work, 2,5,4'-trichlorobiphenyl (IUPAC No. 31) 2,2',3,4'-tetrachlorobiphenyl (IUPAC No. 42), 2,2',4,4',5-pentachlorobiphenyl (IUPAC No. 99), 2,3',4,4',5-pentachlorobiphenyl (IUPAC No. 118), 2,2',3,3',4,4'-hexachlorobiphenyl (IUPAC No. 128), 2,2',3,4,4',5'-hexachlorobiphenyl (IUPAC No. 138), 2,3',4,4',5',6-hexachlorobiphenyl (IUPAC No.168), and 2,2',3,3',4,5,5',6-heptachlorobiphenyl (IUPAC No. 198) were purchased from Ultra Scientific (Kingstown, RI). Compounds were dissolved in absolute ethanol for a 0.1 M stock for cell culture and serial dilutions were prepared in absolute ethanol except congeners 33, 42, 99 and 198 were dissolved in 20% hexane in methanol.

Pesticides were supplied by Axys Analytical (Sidney, BC), except *p,p*'-DDT which was purchased from Sigma (St. Louis, MO).

Hexachlorobenzene (Mr=284.78) was dissolved in diethyl ether for a 10 mM stock solution. Serial ten-fold dilutions were made in DMSO. The range of concentrations tested was 10 µM to 1 nM final in the cell culture media. Hexachlorobenzene was added at 1:1000 (v/v) to cell culture media so that the total solvent content was 0.1%.

β-Endosulfan (CAS# 33213659; Mr=406.9) was dissolved in DMSO to yield a 10 mM stock solution from which dilutions were made in DMSO. The range of concentrations tested was 10 µM to 1 nM allowing β-endosulfan to be added at 1:1000 (v/v) to cell culture media so that the solvent content was 0.1%.

p,p'-DDT (CAS# 50293; Mr=354.49) was dissolved in toluene to yield a 100 mM stock. Ten-fold serial dilutions were made in DMSO so 10 mM *p,p'*-DDT was in 10% toluene / 90% DMSO, 1 mM in 1% toluene/ 99% DMSO, 0.1 mM in 0.1% Toluene/ 99.9% DMSO. The range of concentrations tested was 100 μM to 1 nM with the 100 μM prepared by adding the 10 mM *p,p'*-DDT at 1:100 (v/v) to the cell culture media. Consequently the solvent concentration applied to the cells ranged from 1% (0.1% toluene / 0.9% DMSO) for the highest concentration tested to 0.1% total solvent for all other concentrations.

o,p'-DDT (Mr=354.49) was supplied as a 10 mM stock solution in toluene. Ten-fold serial dilutions were made in DMSO so 1 mM *o,p'*-DDT was in 10% toluene / 90% DMSO, 0.1 mM was in 1% toluene/ 99% DMSO etc. The range of concentrations tested was 100 μM to 1 nM with the 100 μM prepared from 1:100 10 mM stock, Consequently the solvent concentration applied to the cells ranged from 1% (1% toluene) for the highest concentration tested to 0.1% total solvent for all other concentrations.

p,p'-DDD (Mr=320.05) was dissolved in DMSO to yield a 10 mM stock and ten-fold serial dilutions were made in DMSO. The range of concentrations tested was 100 μM to 1 nM with the 100 μM prepared from 1:100 10 mM stock, so for that sample solvent concentration was 1% while for all other concentrations total solvent was 0.1%

p,p'-DDE (Mr=318.03) was supplied as a 7.36 mM stock solution in toluene. Ten-fold serial dilutions were made in DMSO so 1 mM *p,p'*-DDE was in 13.6% toluene / 86.4% DMSO, 0.1 mM was in 1.36% toluene/ 98.64% DMSO etc. The range of concentrations tested was 10 μM to 1 nM with the 10 μM prepared from 1:100 1 mM stock, so for that sample solvent concentration was 1% (0.136% toluene/0.864% DMSO) while for all other concentrations total solvent was 0.1%.

4-Nonylphenol (Acros) (Mr=220.3) was mixed with 20% ethanol in dH_2O to prepare a 1 M stock solution and serial dilutions were prepared in 20% ethanol. The range of concentrations tested was 50 μM to 10 nM and all dilutions were at 1:1000 therefore the final solvent concentration was 0.02% ethanol.

4-Octylphenol was supplied by Axys Analytical (Mr=206.3) as a 100 mM stock solution in 95% ethanol and serial dilutions were prepared in 20% ethanol. The range of concentrations tested was 50 μM to 10 nM and all dilutions were at 1:1000 therefore the final solvent concentrations were < 0.03% ethanol.

Hormones and other ligands were dissolved in absolute ethanol to yield 1 mg/mL stocks from which serial dilutions were prepared. Dihydrotestosterone (DHT), Methyltrienolone (R1881), cyproterone acetate, 17-β-estradiol, and dexamethasone were gifts from Dr. P. Rennie at the B.C. Cancer agency. Cell culture media and reagents were from GIBCO BRL, and other chemicals were purchased from Sigma fine chemicals.

Test compound	**Concentration range**	**Solvent**
Hexachlorobenzene	1 nM to 10 μM	Ether / DMSO
β-Endosufan	1 nM to 10 μM	DMSO
p,p'-DDT	1 nM to 10 μM	Toluene / DMSO
o,p'-DDT	1 nM to 10 μM	Toluene / DMSO
p,p'-DDD	10 nM to 100 μM	DMSO
p,p'-DDE	1 nM to 10 μM	Toluene / DMSO
Aroclor 1254	1 nM to 50 μM	Ethanol
Aroclor 1260	1 nM to 50 μM	Ethanol
PCB congeners	1 nM to 100 μM	Ethanol/Methanol
Nonylphenol	10 nM to 50 μM	Ethanol (50% and 20%)
Octylphenol	10 nM to 50 μM	Ethanol (50% and 20%)
Hormones (DHT, DEX, E, CA, RU486)	0.01 nM to 100 nM	Ethanol (95%)

Table 2-1: Solvent and concentration ranges used for test compounds.

Extensive testing of the effects of the solvents used in the combinations and concentrations described was performed to ensure that they had no effect upon luciferase gene transactivation in the cell culture reporter assay. In pure form ether, DMSO, toluene, methanol and ethanol were all tested with AR, GR and ER systems in all cell lines at 1% and lower concentrations. In addition, solvents added in combination at the following final concentrations was tested with AR, GR and ER systems in all cell lines; 0.9% ether/0.1% DMSO, 0.1% ether/0.9% DMSO, 0.9% toluene/0.1% DMSO, 0.1% toluene/0.9% DMSO, 0.9% methanol/0.1% ethanol, 0.1% methanol/0.9% ethanol. None of these solvent controls produced an effect upon luciferase expression in any of the transiently transfected cell lines.

2.1.2 Sediment Extracts

Sediment Sample Collection
Performed by Environment Canada

Eleven bed sediment samples were collected from three reaches of the lower Fraser River in October 1996: Lytton to Chilliwack (LCH), Fraser River North Arm (NAR) and Fraser River Main Arm (MAN).

Sediment samples were collected using an Eckman dredge (15 cm x 15 cm x 15 cm) at a distance between 0.5 and 5 m from shore. The samples taken from the LCH reach were collected by wading into the river at a depth of 0.5 m to 1.0 m, while those taken from the Fraser River North and Main Arms were sampled entirely by boat. All equipment coming in contact with the sediment samples was made of either stainless steel or Teflon and was solvent washed with acetone and hexane prior to use. A minimum of five bed sediment grabs were collected from each site. The sediment that came in contact with the sides of Eckman dredge was discarded, and only the top layer (2-3 cm) of fine sediment from each grab was collected with a stainless steel spoon. Sediment from each of the grabs was composited in a stainless steel tray and hand homogenized. The homogenized sample was dispensed into Teflon sample containers and kept chilled until frozen. All samples were frozen at –20°C within five hours of collection.

Sediment collected from each site was split into two samples and sent to AXYS Analytical Laboratory in Sidney, B.C. One sample was analysed by gas chromatography and mass spectrometry for trace organic contaminants including PCDDs/furans, chlorophenolics, resin/fatty acids, PAHs, PCBs, pesticides, and 4-nonylphenol (see Appendix 1). Extracts were prepared from the remainder of the sample by the following method for analysis using our cell culture assays.

Raw Sediment Extraction Method

A 5-10 gram sediment was wetted, the pH adjusted to eleven and the sample was shaken with methanol. The aqueous phase was separated from the sediment by centrifuging and decanting, and the aqueous phase was extracted with dichloromethane (DCM). The DCM extract was dried over anhydrous sodium sulphate and set aside.

The wet sediment from above was ground with anhydrous sodium sulphate, loaded into a soxhlet thimble and refluxed with toluene:acetone (80:20) overnight. The dried DCM extract from above was added to the toluene and the combined extract was reduced in volume by rotary evaporation. This resulted in a single extract containing all of the classes of contaminants (PCDDs and furans, PAHs, PCBs and pesticides, chlorophenols and nonylphenols) as well as any coextractives (such as sulphur) that may have been in the sediment. This type of extract is referred to as a "raw" extract, because there is no cleanup or fractionation.

Reach	Location	Sediment No.	Geographical Coordinates
Lytton to Chilliwack	Downstream of Chilliwack	1	49°12.98'; 121°55.90'
	Upstream of Hope	2	49°24.73'; 121°25.35'
	Upstream of Alexandra Bridge	3	49°43.19'; 121°25.68'
	Upstream of Nahatlatch River	4	50°01.09'; 121°31.94'
Main Arm	Ewen's Slough	5	49°06.37'; 123°10.47'
	near Gunn Island	6	49°06.20'; 123°06.86'
	near Western tip of Annacis Island	7	49°09.82'; 122°59.49'
	Annacis Channel	8	49°11.20'; 122°56.39'
	Macdonald's Slough	9	49°13.02'; 123°12.18'
North Arm	Eburne Slough	15	49°12.22'; 123°08.95'
	Fraser River North Arm in Sea Island Channel	16	49°10.77'; 123°10.28'

Table 2-2: Bed sediment sampling site details

Preparation of Sediment Extract Dilutions

Sediment extracts were supplied by AXYS Analytical as two to three mL of a DMSO solution of combined raw extracts of between five and nine g of sediment. These samples were used to prepare two further ten-fold dilutions in DMSO. For application to the cells, four different concentrations of each sediment extract were used. The dilutions and original stock were added at 1:1000 to media (termed 1 x, 0.1 x, and 0.01 x sediment), and for the highest test concentration, the original AXYS stock was added at 1:100 to media (the 10 x sediment). In addition, negative controls (0 sediment) were always prepared in parallel using DMSO added at either 1:100 or 1:1000 to media.

2.1.3 Plasmids

The cell culture transfection assays utilized seven different plasmids. The first plasmid contained the full length cDNA of the rat AR driven by the CMV promoter; the plasmid was prepared at the BC Cancer Agency and has been named pAR6CMV [237]. The rat GR plasmid (prGR) was obtained from Dr. R.J. Matusik (Vanderbilt, Nashville, TS). This construct consists of a 2.8 kb insert containing 24 nucleotides upstream of the rat GR primary translation initiation site to 360 nucleotides downstream of the termination codon and is driven by the double RSV-SV40 promoter [238]. The reporter, also from Dr. R.J. Matusik, that was used for AR and GR assays was the $pARR_3tk$-luc construct, which contains three tandem repeats of -244 to -96 of the promoter region of the rat probasin gene [239]. This promoter region contains androgen response elements to which AR and GR bind and transactivate from *in vitro* (*in vivo*, the probasin promoter is regulated by the AR endogenous gene only). The promoter is ligated in the pT81 vector (American Type Culture Collection, Rockville, MD), which contains a minimal thymidine kinase promoter, and the firefly luciferase gene, as described previously [237]. The firefly luciferase gene is translated to a 61 kDa monomeric protein that does not undergo post-translational modification for enzyme activity and is a genetic reporter that functions immediately upon translation [240]. The human estrogen receptor encoded by pSVMT:wER was a gift from Dr. Carolyn Smith (Baylor, Houston, TX) and has the full length wild type receptor downstream of the metallothionein promoter and simian virus 40 enhancer [241]. ERE-luc was constructed in our lab by Cheryl Portigal and I. The estrogen responsive element (ERE) with the sequence AGCTTCAGGTCACAGTGACCTGA from the salmon vitellogenin gene was inserted upstream of base pairs -109 to 105 of the MMTV promoter in the pMluc plasmid vector [242] (ATCC# 37582).

Two different plasmids were utilised as transfection efficiency controls. Initially pCMV-βGal was used (Clontech, Palo Alto, CA), which expresses the *E. coli* β-galactosidase reporter gene under the control of the human cytomegalovirus immediate early promoter. However, for later studies access to a plate reader luminometer facilitated the use of the pRL-TK transfection control vector, which contains a thymidine kinase promoter upstream of Rluc. Rluc is the slightly modified cDNA encoding Renilla luciferase from the sea pansy *Renilla reniformis* (Promega, Madison, WI). Validation was performed to verify that the change of method for the internal control did not affect the results. This was done by generating hormone standard curves for each reporter assay system. The use of the Renilla as an internal control was preferable since it allows both internal control and firefly luciferase values to be derived sequentially from the same aliquot of cell lysates, eliminating some opportunities for experimental error. Using the β-galactosidase assay necessitated creating two separate aliquots from the cell lysates for the two mutually exclusive assays.

2.2 Methods

2.2.1 Large Scale Plasmid Preparation

A single colony of *E. coli* JM109 (Promega, Madison, WI), transformed with the desired plasmid was picked from a plate and used to inoculate a flask of LB containing ampicillin at 100 μg/mL. Cultures were grown overnight and harvested the next morning (after 16 to 20 hours) by centrifuging the cells at 3,000 x g for 15 minutes at 4°C. The pellet was drained and then resuspended in 5 mL of lysis buffer (per original flask). The cells were transferred to Oakridge tubes and one mL of lysis buffer (50mM Tris HCl pH 8.0, 10mM EDTA) containing 12 mg lysozyme was added and the mixture was incubated at room temperature (RT) for five minutes. Next 12 mL of 0.2 M NaOH, 1% SDS was added and mixed gently and incubated at RT for five minutes or until supernatant cleared, then 7.5 mL of 3 M K+/5 M acetate was added and mixed again by inversion. After incubating on ice for 20 minutes the mixture was spun at 15000 rpm for 15 minutes at 4°C, to yield a solid pellet of cell debris and protein. The supernatant was poured into a 30 mL push-cap tube, five μL of 50 mg/mL RNAse was added and the tube incubated at 37°C for 30 to 60 minutes. The product was transferred into push-cap tubes and an equal volume of phenol / (chloroform / Iso-amyl alcohol [IAA] 24:1 v/v) was added to each tube (8-10 mL) followed by thorough mixing and pelleting of the debris at 5000 rpm for 10 minutes at RT. The upper (aqueous) phase was transferred to a fresh tube and the phenol extraction was repeated before adding an equal volume of Chloroform / IAA (24:1), mixing, and centrifuging for 10 minutes at 5000 rpm. The aqueous phase was transferred to a fresh tube and two volumes of cold 95% ethanol were mixed in before the tubes were incubated at -20°C for 15 minutes. The DNA product was pelleted by centrifugation for

15 minutes at 15000 rpm at 4°C. The supernatant was poured off; the pellet was washed with 70% ethanol and drained. Each pellet was dissolved in 600 μL of dH_2O, transferred to a 1.5 mL microcentrifuge tube and 150 μL of 5 M NaCl was added plus 750 μL of 13% PEG. The DNA was pelleted by microcentrifuging for 15 minutes at 4°C then washed with 70% ethanol, drained, air dried and finally resuspended in 300 μL TE (20mM Tris, pH 7.8, 1 mM EDTA). Plasmid purity and concentration were determined before use by measuring optical density at 260 and 280 nM and by running samples on 1% agarose gels [243].

2.2.2 Mammalian Cell Culture

Four human derived cell lines were used.

PC-3 cells (obtained from Dr. P.S. Rennie at the BC cancer agency) initiated from a bone metastasis of a grade IV prostatic adenocarcinoma from a 62-year-old Caucasian male [244]. The cells exhibit testosterone-5-α reductase activity, lack endogenous AR and are androgen insensitive. They were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco BRL, Burlington, ON) + 5% fetal bovine serum (FBS), for assays with glucocorticoid and androgen receptors.

LNCaP cells (obtained from Dr. Martin Gleave at the BC cancer agency) originated as a lymph node metastasis from the prostate in a 50 year-old Caucasian male. These cells are well-differentiated androgen-sensitive prostate cells, which express an endogenous mutated yet functional AR with an alanine to threonine mutation at position 868 [245]. LNCaP cells were maintained in RPMI 1640 defined medium (Gibco BRL, Burlington, ON, Canada) supplemented with 5% FBS under standard conditions (37°C, 5% CO_2) for assays with androgen receptors.

HepG2 (ATCC, Manassas, VA) is a human hepatocellular carcinoma line cultured in Minimum Essential Medium (MEM; Gibco BRL, Burlington, ON) + 5% FBS and used for assays with the estrogen receptor and ERE-luc [246].

HeLa AR-FLAG (Dr. M Carey, UCLA, CA) is a cervical derived adenocarcinoma cell line which has been stably transfected with human wild type androgen receptor with an N-terminal FLAG-tag, cultured in DMEM with 10% FBS [247].

General Culture

All cell lines were grown in an adherent manner on 175 cm^2 plates until confluent before passaging by trypsinisation. Passage number was noted and new cells were brought up from liquid nitrogen stocks as required.

Transfection methods

Different methods of transfection were attempted with the cell lines used to determine the optimal for each. After comparing results from experiments using CO_2 stress, calcium phosphate alone, and calcium phosphate followed by glycerol shock it was determined that the latter method was the best for both PC-3 and HepG2 cell lines. However, when later work was commenced with LNCaP cells all of these methods proved unsatisfactory and so a commercial transfection reagent (Lipofectin; Gibco BRL/Invitrogen, Carlsbad, CA) was tested and found to yield higher transfection efficiency in LNCaP. Subsequent comparison in PC-3 cell transfection showed no difference between calcium phosphate followed by glycerol shock and Lipofection. Consequently, Lipofection was used with both prostate cell lines for latter experiments.

Calcium Phosphate / Glycerol Shock Transfection Method

The transfection method used for many of the PC-3 (AR or GR) and all the HepG2 (ER) experiments was based on the Ca_2PO_4 / glycerol shock method [248]. This method was found to be very inefficient in LNCaP cells and was not used with this cell line.

For transfections, PC-3 or HepG2 cells were cultured in media supplemented with 5% hormone stripped (with dextran coated charcoal) fetal bovine serum (S-FBS) at a density of 3 x 10^5 cells per well in six well cell culture plates or half that number per well in 12 well plates (Costar, Corning NY) and incubated overnight under standard conditions. To transfect a six or 12 well plate the following mix was prepared: 18.75 μL 2 M $CaCl_2$, 1.5 μg reporter construct, 3 μg receptor construct, 0.5 μg control plasmid (pCMV-βgal or pRLTK) and sterile dH_2O to bring the volume to 156.25 μL. Finally 156.25 μL HEPES buffered saline was added and the mixture incubated at RT for 30 minutes. The media was aspirated off the cells and fresh media was added followed by the DNA mix (25 μL per well on 12 well plates, 50 μL per well on six well plates) and the plates were incubated for four to six hours. After the plasmid DNA had precipitated onto the cells, wells were drained and shocked with 20% glycerol in media (250 μL per well for 6 well plates) for one minute before 750 μL phosphate buffered saline (PBS) was added and wells drained, washed with another 500 μL PBS and drained again. Media containing test compounds over a range of concentrations was added to each well (2 mL for each well on a 6-well plate). Three to six wells were used for each concentration of every test solution. Known agonist for the receptor under investigation was added to one set of tests to enable the detection of antagonist activity. The two highest concentrations of a test compound or extract were assayed without the addition of known agonist to assay for agonist activity. Cells were incubated with the test mixture for 20-24 hours before their health was visually assessed and they were harvested.

To harvest the cells, wells were drained, washed with PBS, drained again and PBS containing 1 mM EDTA was added. After 15 minutes the cells were washed off the substratum and transferred to 1.5 mL tubes. Cell pellets were spun down gently (4000 rpm, 2 minutes) and the PBS was aspirated off. Each pellet was resuspended by vortexing in 50 μL 100 mM Tris pH 7.5, 0.1% Triton-X-100. Pellets were frozen and thawed to aid lysis and cell debris was pelleted by spinning at 15000 rpm for 10 minutes in a microfuge. The resultant supernatant was assayed for luciferase and β-galactosidase activities.

Lipofection

Six, 12 or 24 well plates were seeded as described above and plasmids were cotransfected into the cells using LIPOFECTIN reagent (Gibco BRL, Burlington, ON, Canada). Cells are cultured overnight in RPMI 1640 supplemented with 5% hormone stripped (with dextran coated charcoal) fetal bovine serum (S-FBS) at a density of 1.5x 10^5 cells/well in 12 well cell culture plates (1ml/well) before transfection.

To each plate was added 1.5 μg of the receptor plasmid (AR, ER or GR), 1 μg of reporter pARR3tk-luc or pERE-luc and 0.5μg of pCMVβ-gal or 0.02 μg of pRL-TK. To transfect a batch of twelve 6- or 12-well plates 360μL Lipofectin was added to 3.6ml RPMI (serum free). Next, the DNA was added to a separate 3.6ml aliquot of RPMI (serum free). After 30-45 minutes at room temperature the two mixtures were combined, and then incubated for a further 10-15 minutes at room temperature. The total volume was made up to 150 mL and then the mixture was aliquoted onto the aspirated plates of cells (1 mL per well of 12-well; 2 mL per plate for 6-well format). After overnight incubation hormone and test compound were added to 5% DCC serum RPMI (12-well 1ml per well). The plates were drained by aspiration and hormone /compound in media was added.

Cells were harvested 48 hours later in PBS with 1 mM EDTA and spun at 4000 rpm at 4°C for two minutes. Supernatant was removed and 50 µL Passive Lysis Buffer (Promega, Madison, WI) was added. Cells remained on ice for 15 minutes and were then vortex mixed and frozen at –80°C until analysis.

2.2.3 Reporter Gene Assays

In preliminary testing it was determined that PC-3 cells exhibited poor responsiveness to estrogens even when transfected with ER and ERE-luc. Consequently, all ER assays were performed in HepG2 cells. All GR assays reported here were performed in PC-3 cells. AR assays in most cases were performed initially in PC-3 cells and verified by repeating in LNCaP cells.

Dual Luciferase Assay

Luciferase assays were performed using the luciferase assay kit (Promega, Madison, WI) or the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). Transfected cell lysates in passive lysis buffer (Promega, Madison, WI) were spun at 5000 rpm for 5 minutes to pellet cell debris and 10 µL of sample supernatant was mixed with 50 µL of firefly luciferase reagent and light emitted was measured in a liquid scintillation counter (LSC) or the EG&G Berthold Microplate Luminometer LB 96V. Samples measured using the scintillation counter were read in sets of three such that each sample was mixed for two minutes before entering the counting chamber. Examples of standard curves generated are shown in Figure 2-1. Luminescence measured is proportionate to concentration of luciferase enzyme in the supernatant, and is therefore a measure of luciferase expression in the transfected cells.

The luciferase assay was validated using pure firefly luciferase enzyme (Promega, Madison, WI). The response was linear over a range from 0.01 mg/mL to 1 µg/mL before levelling off at a saturating response. None of the cell culture assay samples tested came within and order of magnitude of this saturation level. The purified enzyme was also used to compare different batches of luciferase reagent and confirm the manufacturers assertion that no significant variation occurred between batches.

β-Galactosidase Assay

Thirty microlitres of each cell extract was added to a microcentrifuge tube containing 20 μL Mg solution (15 mM $MgCl_2$ and 682 mM β-mercaptoethanol) and 200 μL 0.1 M NaH_2PO_4 pH7.5. Fifty microlitres of o-nitrophenylguanidine (5.5 mg/ml in 0.1 M NaH_2PO_4 pH7.5) was added and the tubes incubated at 37°C for between 30 minutes to overnight until yellow colour developed. Reaction was stopped with 500 μl of 1 M Na_2CO_3, and the optical density of each tube was measured at 420 nM [243].

Chloramphenicol Acetyl Transferase (CAT) Activity Assay

To determine the level of expression of the androgen driven reporter gene from the LPB-CAT transgenic mice their prostates were placed on the tip of a chilled Dounce pestle and homogenised in 300 μL lysis buffer (0.1 M Tris pH 7.8, 0.1% Triton X-100). Homogenate was transferred to 1.5 mL Eppendorf tubes and tissue debris was pelleted by centrifugation for three minutes at 8000 rpm at 4°C. The supernatant was collected in a new tube and the protein concentration determined using a Pierce BCA (bicinchoninic acid) assay kit (Pierce, Rockford, IL) on samples in 96 well plates.

A 5 μg/mL dilution was prepared in buffer and heated at 65°C for 10 minutes then cooled to RT for 10 minutes. 200 μL of each sample was added in duplicate to scintillation vials and 75 μL of reaction mix (7μM ^{3}H-acetyl CoA, 3.33 mM chloramphenicol, 0.1 M Tris pH 7.8) was added to each. A 3 mL layer of Scintilene cocktail (Fisher) was overlaid and the reaction was left to go for 30 minutes. The released activity was counted in a scintillation counter over eight cycles and the slope of the straight part of the resultant curve for each sample was calculated.

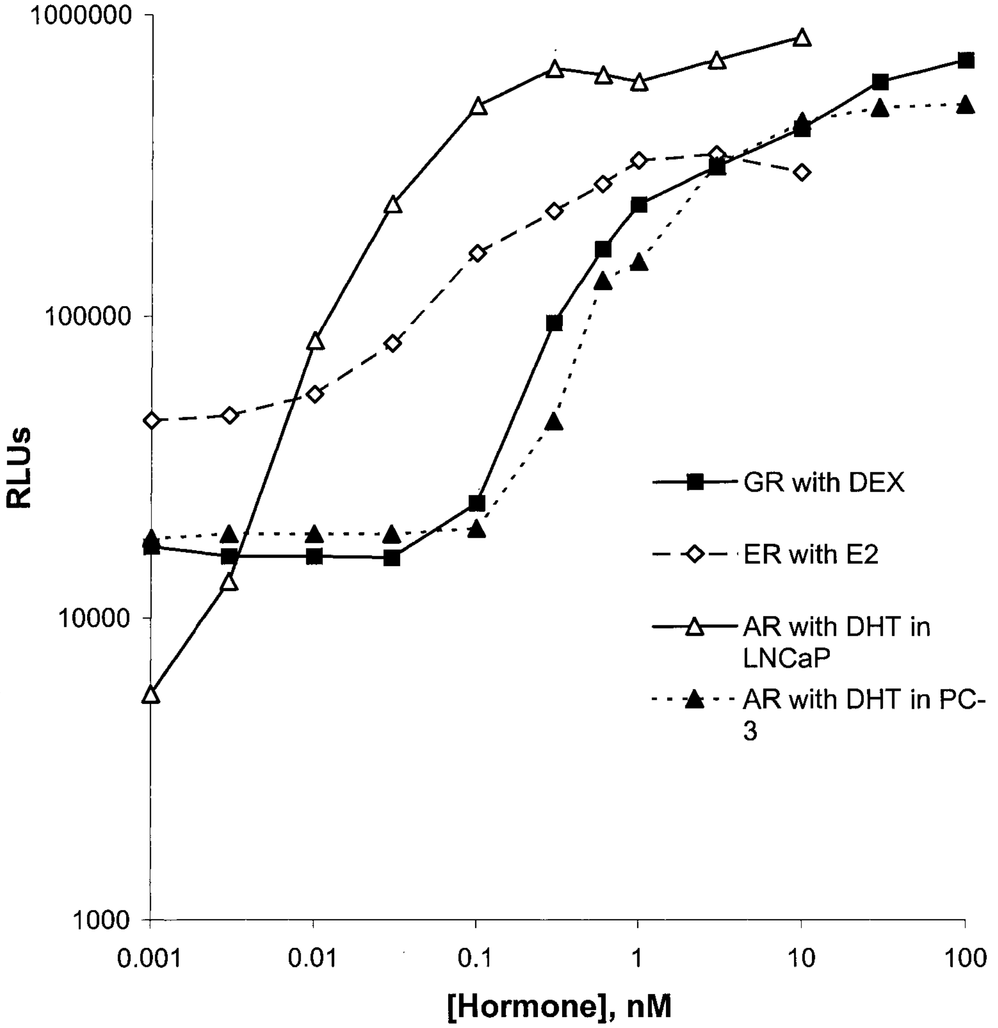


Figure 2-1: Representative hormone response curves for the luciferase reporter gene assay. Cells transfected with steroid receptor and corresponsding luciferase reporter constructs (ARR3-luc for AR and GR, or ERE-luc for ER) were exposed to increasing concentrations of known agonist fro 24 hours. Cell lysates were assayed for luciferase levels induced and normalised to internal transfection control. Cell lines used were LNCaP or PC-3 for AR, PC-3 for GR and HepG2 for ER.

2.2.4 Ligand Competition Experiments

FLAG-AR HeLa Cell Competitive Binding Assay

HeLa cells expressing a stably transfected FLAG-tagged AR were obtained from Dr. M. Carey (UCLA, CA) [247]. A confluent 175 cm^2 flask of cells was used to seed 24 six cm Petri dishes at 1.5 x 10^5 cells/ml in DMEM /10% S-FBS which was then incubated for four to six hours. The media was changed to DMEM supplemented with 10% S-FBS, 0.3 nM [1,2,4,5,6,7-3H(N)]-DHT (NEN Life Science Products, specific activity=123 Ci/mol, initial concentration=1 mCi/ml) and test substances were added in 100 μL DMEM /10% S-FBS. The cells were incubated for 20 to 24 hours before harvesting by washing off with PBS containing 1 mM EDTA and transferring cells to 1.5 mL microcentrifuge tubes. Nuclear extracts [249] were prepared and frozen at -80°C prior to use. Extracts were thawed then incubated for 6 hours with 10 μl of agarose beads conjugated with FLAG monoclonal antibodies (Sigma) at 4°C for 6 hours. Beads were washed twice with buffer D containing 20 mM HEPES pH 7.9, 20% glycerol, 0.3 M KCl, 0.2 mM EDTA, 0.05% IGEPAL®, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and then resuspended in buffer D. Resuspensions were measured for levels of tritium in a Beckman LS 6500 scintillation counter, and results were expressed as mean ± standard equivalent of the mean (SEM).

PC-3 Cell Competitive Binding Assay

PC-3 cells were seeded onto 6 or 12 well plates and transfected with pAR6CMV or prGR as described for transcriptional activation assays above. Radiolabeled hormone was added to 1 nM final concentration (approximately 2 to 5 kBq) and the cells were incubated for three to six hours before adding unlabeled test competitor at a range of concentrations. After 16 - 20 hours the supernatant was collected and the cells washed three times with PBS. The radiolabel associated with the three fractions (i.e. supernatant, wash, and cell) was counted in a liquid scintillation counter (LSC) to give a measure of ligand displacement from cellular binding sites by the test compound.

Recombinant AR-Ligand-binding Domain Competitive Binding Assay

Recombinant rat AR-LBD (PanVera [1.390 mg/ml]) with an amino acid sequence identical to that of the human AR-LBD was used with a hydroxylapatite assay to determine the binding displacement of ^{3}H-R1881 (Methyltrienolone; NEN Life Science Products, 75.2 Ci/mMol, 1 mCi/ml) in the presence of 100 nM-100 μM PCB congener. Materials and method were used as outlined by PanVera with a 33% Macro-Prep Ceramic Hydroxylapatite Type I 40 μM (BioRad) slurry, binding and wash buffers. The final AR-LBD concentration was 1 μg /ml in the assay mixture. Microfuge tubes were centrifuged briefly after the addition of AR-LBD and ^{3}H-R1881/binding buffer, as well as after the addition of PCB congener, to ensure mixing. Tubes were then incubated overnight at 4°C. Following incubation, 100 μL of 33% hydroxylapatite slurry was added, vortex mixed and incubated on ice for 10 minutes. Vortexing was repeated twice while incubating to resuspend the hydroxylapatite. One millilitre of wash buffer was added to each tube, vortex mixed and microfuged for 1 minute at maximum RPM. Supernatant was carefully aspirated and the wash was repeated twice more. The washed hydroxylapatite pellet was resuspended with 200 μL of 95% ethanol and transferred to scintillation vial. The tube was washed with a further 200 μL of 95% ethanol and combined with the hydroxylapatite pellet. Four millilitres of Scintiverse scintillation fluid was added and each vial was measured for levels of tritium in a Beckman LS 6500 scintillation counter. Results are expressed as pmole bound ^{3}H-R1881 per mg AR-LBD protein. Values were adjusted for non-specific binding determined by adding excess cold R1881.

2.2.4 *In vivo* Animal Experiments

Rearing

This study used CD1 transgenic mice possessing the androgen sensitive LBP-CAT reporter gene [250]. This transgene is comprised of a large fragment of the rat probasin promoter (-11500 to +28 bp) (LPB) linked to a chloramphenicol acetyl transferase (CAT) gene to achieve highly prostate-specific expression of CAT in the prostatic secretory epithelial cells. Expression is highest in the ventral prostate, and therefore, this lobe of the prostate was used for biochemical assays. The LPB-CAT transgenic mice were raised in the core facility at the Jack Bell Research Centre according to the University of British

Columbia (UBC) and Canadian Council on Animal Care guidelines. For each study, mice were paired as close in age as was possible, with one male and one female per cage. The females were dosed with test compound in 100μL corn oil daily on a continual basis starting on the day of pairing until the pups were weaned at 21 days of age. After weaning the pups were dosed directly. For the first 7 days they received a half dose (100 μL) and from 28 days of age onwards they received a full dose. Dosing was by oral gavage using 200 μL as a regular dose volume of test compound prepared in canola oil.

Dissection

Mice were anaesthetised with Halothane (MTC Pharmaceuticals), cardiac puncture was performed to obtain whole blood (typically 1 to 2 mL per animal) and the halothane dose was increased to euthanise the animal. Dissections were performed upon each individual animal before the next mouse was anaesthetised. The body cavity was opened and organs removed and weighed before being frozen in dry ice or fixed in neutral buffered formalin. Liver, kidney, heart, thymus, adrenal, and brain (separated into hypothalamus, cortex and cerebellum) were removed from each animal. In addition testes, seminal vesicles, epididymis and prostate were removed from males, and ovaries and uterus plus fallopian tubes from females.

Histology

Tissues were fixed in 10% neutral buffered formalin for 24hours then stored in 70% ethanol before being embedded in paraffin wax. Slides were prepared from the paraffin embedded tissues by the Vancouver General Hospital pathology department. Histological assessment was performed by UBC veterinarian Dr. Stephane Lair on prostates, livers, epididymides and testes of PCB 42 and nonylphenol treated mice.

Hormone Assays

Total serum T4 was measured using the ELISA kit from Monobind Inc. (Costa Mesa, CA). Reference samples provided by the manufacturer were used to derive a standard curve from which values for the mouse serum samples could be determined. Standards and samples were assayed in duplicate and the assay plate was read at 450nM using a 620nM reference wavelength to correct for well imperfections or turbidity.

Normal human values are 76 ± 16 ng/ml for male, 82 ± 17 ng/ml for females. Serum testosterone levels were measured by the BC Cancer Agency using radioimmunoassay.

2.2.5 Other Methods

Data Analysis

Firefly luciferase data was correlated with *Renilla* luciferase or β-galactosidase internal control results (where available) to correct for transfection efficiency. The results from each set of samples were averaged and plotted against concentration of test sample. One-way analysis of variance (Microsoft Excel or Instat software) was used to determine the significance of any change in luciferase activity ($p<0.05$). Pairwise comparisons were performed using Students T-test to determine which samples were significantly different from the control. If a significant increase in firefly luciferase activity was identified between control and a test sample then the compound was identified as an agonist. Conversely if there was a significant decrease in firefly luciferase activity between control and a test sample then the compound was identified as an antagonist. Any other outcome is listed as no effect unless specifically noted. Most experiments were repeated at least three times, however, only one complete set of data was obtained for some of the sediment-receptor combinations. Therefore, for these experiments the stated activity in a given sediment extract is sometimes based on the results of a single experiment.

Sodium-Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

For the ligand competition assays using FLAG-tagged AR expressed in stably transfected HeLa cells it was necessary to normalise for AR levels between samples. To do this quantitative Western blotting was used. SDS polyacrylamide gels were prepared as described by Neville [251] using the BIO-RAD mini-protean slab gel casting system. A 12% separating gel and a 3% stacking gel were used unless otherwise stated. Hela cell lysates were prepared in protein sample buffer (2% SDS, 5% β- mercaptoethanol, 10% glycerol, 27 mM H_2SO_4, 55 mM Tris-HCl pH 6.1 with bromophenol blue) and boiled for three minutes before being loaded onto the gel. Gels were run at 16 mA constant current until the dye front reached the separating gel, then at 40 mA constant current until the dye front reached the bottom of the gel. Gels are run at 60 V maximum through the stacking gel and160 V maximum thereafter. Proteins were transferred to nitrocellulose, as described below.

Western Blot

Western blotting was performed according to the method of Harlow and Lane [252] to confirm levels of FLAG-tagged AR present in nuclear extracts. Proteins separated on SDS-PAGE were transferred to PVDF membranes, which were then blocked with 5% (w/v) skim milk in PBS-T for at least 45 minutes at room temperature, or overnight at 4°C. All subsequent incubations were performed at room temperature on a lab shaker, unless otherwise noted. The androgen receptor band was visualized as follows: the membrane was incubated with mouse monoclonal antibody to human AR DBD (Pharmingen) in PBS-T, given three five minute washes with PBS-T and then incubated with 1:4000 solution of Amersham goat anti-mouse IgG horseradish peroxidase for 60 minutes. The blots were washed eight times in PBS-T, three times for five minutes, twice for 15 minutes then a further three times for five minutes, before bands were detected using ECL™ reagent (Amersham Pharmacia).

Films from Western blots were analysed using PDI Quantity One Quantitation Software version 4.2.1 (BioRad) to determine and compare band densities for nuclear extracts from each competition assay. Statistical significance for this section was calculated using one-way analysis of variance ($p<0.05$).

Chapter III - Pesticides and Complex Mixtures

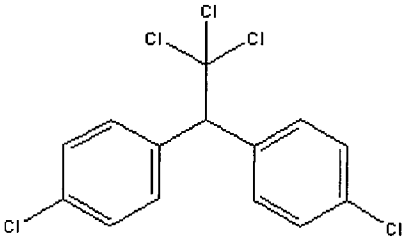
A selection of pesticides, pulp and paper mill by-products, and sediment extracts from the Fraser River were tested for their effect on androgen, glucocorticoid and estrogen receptor mediated gene transactivation. The pesticides tested (Figure 3-1) were the components of technical grade DDT (*o,p*'- and *p,p*'- isomers plus *p,p*'-DDD) and the metabolite (*p,p*'-DDE), plus two additional pesticides (hexachlorobenzene and β-endosulfan). Pulp and paper mill samples were obtained from three mills, two in British Columbia and one in Ontario. Analysis of the sediment extract for a variety of organochlorine and hydrocarbon contaminants was performed by Axys Analytical laboratories (Sidney, B.C.).

3.1 Hexachlorobenzene

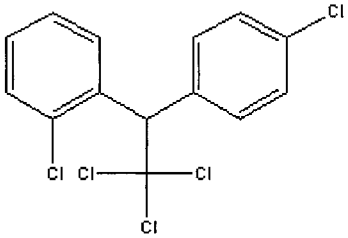
Hexachlorobenzene (HEX) was used primarily as a selective fungicide against bunt (*Tilletia tritici*) and smut particularly for the treatment of wheat, onion, sorghum, safflower and sunflower seeds as well as against certain insect pests and wireworms. In addition HEX was used as a chemical intermediate for dye and hexafluorobenzene manufacturing. In Europe, though not in North America, HEX has been used as the precursor for the wood preservative pentachlorophenol and in the production of aromatic fluorocarbons used to impregnate paper. European pentachlorophenol made by alkaline hydrolysis of HEX has a higher content of polychlorinated dibenzo-p-dioxin and dibenzofuran impurities than its North American equivalent. In addition HEX was used in organic syntheses for example, as a raw material for synthetic rubber; as a plasticizer for polyvinyl chloride; and as an additive for pyrotechnic compositions for the military.

During the 1970s the use of HEX as a fungicide was banned in the United States, Canada and some European countries. Hexachlorobenzene is still present as an impurity in the pesticides pentachlorophenol, dacthal, atrazine, picloram, pentachloronitrobenzene, chlorthalonil, and lindane [253]. The major global sources of HEX contamination today are combustion processes and continued use as a pesticide in many jurisdictions.

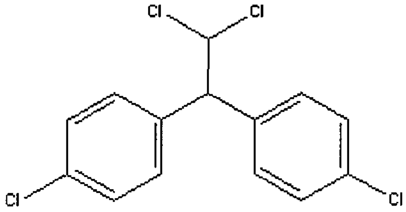
p,p'-DDT



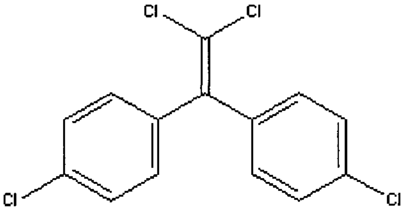
o,p'-DDT



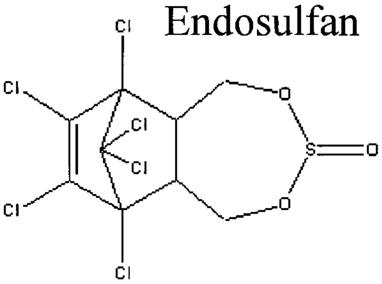
p,p'-DDD



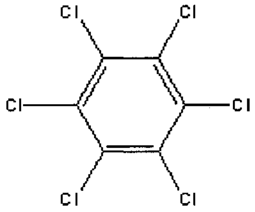
p,p'-DDE



Endosulfan



Hexachlorobenzene



2,3,7,8-tetrachlorodibenzo-p-dioxin

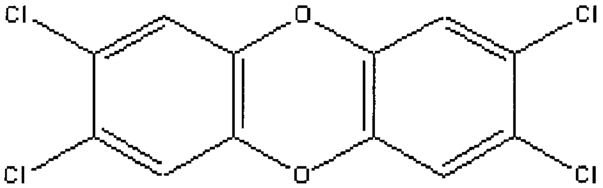


Figure 3-1: The structures of the pesticides in this study and of 2,3,7,8-tetrachlorodibenzo-p-dioxin

3.1.1 Environmental Fate and Exposure

Hexachlorobenzene is very persistent and binds tightly to soil and sediments, properties that, in combination with a low aqueous solubility prevent HEX from readily leaching into water. The estimated half-life in soil is three to six years and in the atmosphere is two years. HEX is relatively volatile and consequently long-range atmospheric transport of HEX to the Arctic and other remote areas is a well-recognised phenomenon. The substance has been detected in Arctic air, snow, seawater, vegetation and biota. It had also been observed in other remote areas such as the North Pacific Ocean and in the rainfall of two remote islands on Lake Superior. HEX has been measured in freshwater and marine biota around the world, including grass shrimps, sheephead minnows, and pinfish. Concentrations of HEX have been observed in fish-eating birds and predatory bird species [253].

HEX is a bioaccumulative substance with bioconcentration factor (BCF) values in the range from 375 to >35,000 [253]. HEX accumulates in human body tissues and breast milk and as a result of its widespread use and environmental persistence it is found in the majority of human adipose samples tested [254, 255]. In a study of breast-fed Australian infants, 27 percent were found to have a dietary HEX intake exceeding the World Health Organisation (WHO) average daily intake standard of 0.6 μg/kg/day [256, 257]. In Canada, levels of HEX in Inuit mother's milk (136 ng/g) are up to nine times higher than levels seen in mothers' milk in Southern Quebec [258].

Food intake is the primary source of exposure of the general population. HEX residues have appeared in a wide variety of foods and have been commonly found in meat, milk and eggs. The average dietary intake in the USA in the years 1978 to 1982 was estimated to be below 0.01 μg/day although one Japanese study estimated intake in that country to be 0.5 μg/day in 1977. Due to the accumulation of HEX in breast milk, intake by nursing infants is higher than for adults: breast-fed infants in Australia and Norway were estimated to consume up to 40 μg/day in the mid-1970s. Levels in drinking water were found to average 0.1 ng/L in one study in Canada [259].

3.1.2 Health Effects

Due to its liver toxicity, acute high dose exposure to HEX is associated with *porphyria cutanea tarda,* normally a disorder resulting only from a congenital defect.

This disease can cause red-coloured urine, skin sores, change in skin colour, arthritis, and problems of the liver, nervous system, and stomach. In Turkey between 1955 and 1959, some 5000 people were exposed to HEX when treated seed grain was used to make flour. Some 600 cases of *porphyria cutanea tarda* were reported. It was estimated that the affected individuals had consumed 50-200 mg/day over a period of weeks [253, 260]. Although most recovered after exposure ceased, some continued to experience abnormal porphyrin metabolism for at least 20 years after the initial exposure [261]. Children born to mothers known to have ingested HEX-tainted food during pregnancy experienced acute illnesses and rashes. High miscarriage and infant mortality incidence was observed, over 90 per cent in some villages [261, 262]. Children exposed to contaminated bread had short stature, atrophied hands and fingers, osteoporosis and arthritic changes. Follow up studies reported *porphyria cutanea tarda*, reduced growth, and arthritic symptoms in children directly exposed to contaminated bread or mothers' milk [261, 262]. There was also a 35 percent prevalence of enlarged thyroids as has been reported in other incidences of accidental HEX exposure and in laboratory studies on rats [263-268].

Doses of HEX as low as 0.01 mg/kg/day have affected the reproductive tissues in female monkeys, particularly the ovary [259, 269, 270]. Several reports have also indicated HEX exposure can impact steroidogenesis [263, 271]. Placental and lactational transfer of HEX, demonstrated in a number of species, can adversely affect both the foetus and nursing offspring [272-275]. Adverse effects on suckling infants are generally observed more frequently, and at lower doses than embryotoxic or fetotoxic effects. This likely reflects the apparent accumulation and concentration of HEX into breast milk from serum and adipose reserves [276, 277].

3.1.3 Results and Discussion

Hexachlorobenzene was tested for androgenic or antiandrogenic activity in PC-3 and LNCaP cells. Jody Saito performed experiments in LNCaP cells. In both cell lines androgenic activity was repeatedly seen but only in the presence of DHT (Figure 3-2A and Figure 3-3). No androgenic activity, in the form of increased firefly luciferase activity, was detected when HEX alone was added to the PC-3 cells.

These results demonstrate a previously unreported androgenic activity for HEX in human cells. This activity is only seen when HEX is added in combination with a known

AR agonist, and in competitive binding experiments HEX was unable to displace agonists from the AR ligand-binding site. The underlying mechanism for the additive agonist activity is unclear and further work will be required to determine what other point in the gene regulatory pathway the promotion of androgen mediated gene transactivation is occurring. This activity could play a role in the reported effects of HEX upon the reproductive tract, although since these effects are mostly seen in the ovary the underlying mechanism is not clear [259, 269, 270].

Although it has been demonstrated that HEX affects thyroid hormone homeostasis [267, 268] and that thyroid hormone can in turn promote expression of AR [278, 279] this is unlikely to be the mechanism of increased androgenicity seen in our tissue culture assay. The androgen receptor in this assay is under the control of a constitutive promoter, not its normal inducible one, and the proposed mechanism of thyroid disruption *in vivo* is via increased liver metabolism [280].

When HEX was tested for interaction with GR in both PC-3 and LNCaP no significant activity was detected as determined by changes in firefly luciferase activity (Figure 3-2B). Similarly when HEX was tested for estrogenic or antiestrogenic activity no significant change in firefly luciferase activity resulted from the addition of HEX either in the presence or absence of 17β-estradiol (Figure 3-2C).

The lack of evidence for activity with the estrogen receptor seems to indicate that this receptor pathway is not responsible for the toxic effects of HEX, however our assay made use of a normally non-estrogen responsive cell line (HepG2) in a relatively rudimentary system and more complex assays particularly *in vivo* ones for estrogenic activity, such as the utertorophic assay, may yield very different results, as has been shown for some other test compounds [281-285]. The lack of activity with the glucocorticoid receptor indicates that the reported effects of HEX upon the immune system are not likely mediated through GR (Figure 3-2C).

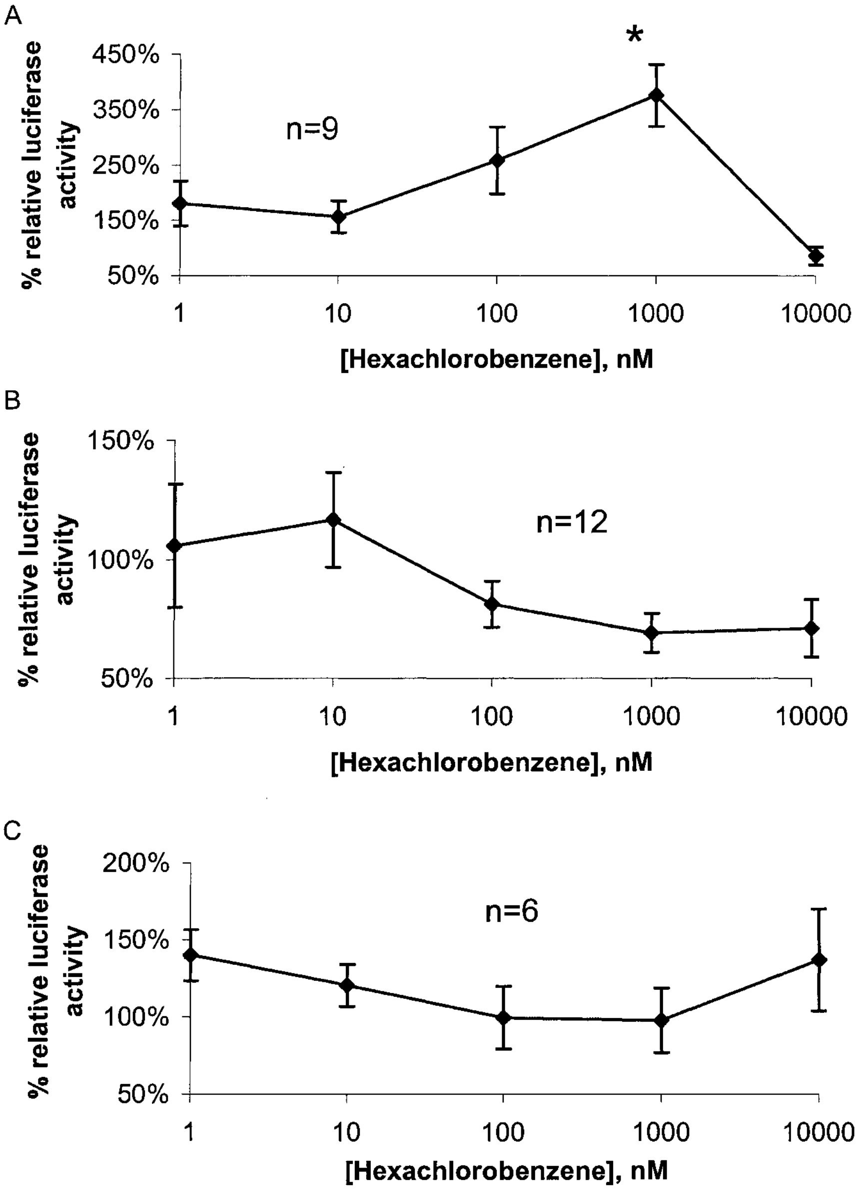


Figure 3-2: The effects of hexachlorobenzene on receptor mediated luciferase transactivation. On AR with 1nM DHT in PC-3 cells (A); on GR with 1nM DEX in PC-3 cells (B); on ER with 1nM E2 in HepG2 cells (C). The * indicates value is significantly different ($p<0.05$ using Student's T-test) from control (no test compound added, set at 100% relative luciferase activity) . Number of individual wells (n=), from multiple experiments, used to derive the graph is shown. Error bars indicate the standard error of the mean (SEM).

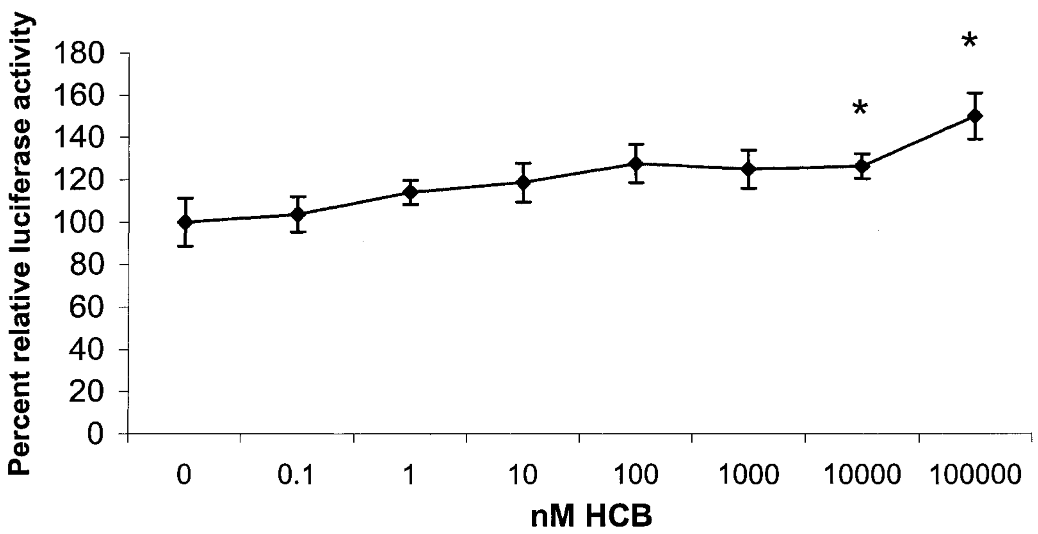


Figure 3-3: The effects of hexachlorobenzene on androgen driven gene transcription in LNCaP cells in the presence of 0.05nM DHT. The * indicates a value is significantly different (p<0.05, using Student's T-test) from control (no test compound added, set at 100% relative luciferase activity). Error bars indicate the standard error of the mean.
These experiments were performed by Jody Saito.

3.2 β-Endosulfan (END)

Commercially sold as a mixture of α and β isomers under the name Thiodan, endosulfan is an insecticide used on deciduous fruits, nuts, cotton, leaf crops & vegetables to control a wide array of insects including the Colorado beetle and aphids. Non-agricultural uses include the control of termites and tsetse fly. Currently its use is controlled in most Western countries and banned in some, but remains widespread in much of the developing world, particularly Asia and South America [286].

3.2.1 Environmental Fate and Exposure

Endosulfan is hydrolysed within weeks in most aqueous environments to endosulfan diol [286]. Breakdown is promoted by availability of oxygen, increasing pH and temperature. In soil, endosulfan breakdown can occur by photolysis at the surface, hydrolysis or oxidation, or metabolism by micro-organisms. The soil half-life in sandy loam has been reported at between 30 and 800 days [287] with β-endosulfan being more persistent than the α-isoform.

The major metabolic pathway for endosulfan was determined to proceed through endosulfan sulphate, endosulfan diol and endosulfan alpha-hydroxyether and finally to endosulfan lactone [286]. The photolysis of endosulfan yields two photo-metabolites, endosulfan diol and endosulfan alpha-hydroxyether. In addition when exposed to sunlight on plant leaves, alpha-endosulfan undergoes isomerization to the more stable beta-isomer [288, 289]. Endosulfan sulphate retains much of the toxic effects of the parent compound but is resistant to environmental breakdown although it can be metabolised or hydrolysed in the presence of hydroxyl-radicals.

Almost all studies of endosulfan concentrations in the environment have failed to identify contamination or found low levels present. Typically concentrations in water including samples from agricultural areas are below 1ppb, and in soils and sediments 1ppm or lower [286].

3.2.2 Human Exposure

Since it is readily metabolised, endosulfan does not appear to accumulate in plants and animals [286]. With a bioconcentration factor of 2755, endosulfan is considered as having moderate potential for bioaccumulation [290, 291]. The major source of human exposure is from food recently treated with endosulfan or a pesticide formulation containing it. Dermal exposure is another potential route of entry in occupationally exposed individuals. Total endosufan levels vary greatly in foods but were as high as 0.55 mg/kg in tomatoes and 1.1 mg/kg in pears [292-294]. Estimated total of alpha and beta-endosulfan plus endosulfan sulphate intake from food was 1.18 μg/day as determined by averaging the average daily intake values for the years 1971-76 [286, 295].

3.2.3 Health Effects

In humans numerous fatalities have been reported from both deliberate and accidental exposure to endosufan and endosufan-containing preparations [286]. In most cases it was impossible to determine the precise dose, but in one case a 43 year old male intentionally ingested 260 mg/kg in a single dose [296]. The patient died within 4 days from cerebral edema and herniation.

Endosulfan has been shown to have a fairly high general toxicity and an LD_{50} for mice has been reported as low as 7.5 mg/kg [297]. In many studies vomiting is induced by oral intake, and fatality is restricted to those animals that fail to purge the endosulfan. Studies of chronic toxicity at levels below this are limited but have identified three primary sites of impact; the kidney, liver and testes.

Renal haemorrhage and tubular necrosis have been reported in numerous studies of the effects of endosulfan on animals [286]. In both dogs and rats dosage of 10 mg/kg/day was sufficient to induce damage [298, 299]. Endosulfan has been shown to preferentially accumulate in the proximal tubules of the kidneys when administered at low dose over a prolonged period [290]. This creates localised yellow pigmentation. Increased liver weight has been shown in multiple studies and is associated with increased expression of several liver enzymes including alanine and aspartate aminotransferases and alkaline phosphatase [286].

Endosulfan seems to produce substantial effects upon the testes in several species. Early studies identified decreased testicular weight and apparent degeneration of seminiferous tubules in rats after endosulfan exposure [297]. Increased serum testosterone (T) was later reported in male rats dosed with 7.5 and 10 mg/kg/day [300]. In a follow up experiment they showed that these doses also caused a decrease in testicular T concentration and declines in serum LH and FSH levels [301]. In an investigation of the impact of 2.5 mg/kg/day endosulfan on testicular histology in rats it was shown that spermatogenesis decreased and consequently sperm counts fell [302] and that younger animals were more sensitive to these effects [303, 304]. Interestingly, multigenerational breeding studies have shown no impact of endosulfan on rat fertility [286].

3.2.4 Results and Discussion

As described in the Materials and Methods β-endosulfan (END) was tested for androgenic or antiandrogenic activity in PC-3 cells transfected with AR. No androgenic activity, in the form of increased firefly luciferase activity, was detected either in combination with DHT (Figure 3-4A) or when END alone was added to the cells. β-Endosulfan also did not exhibit any consistent antiandrogenic activity in this assay as there was no suppression of firefly luciferase expression in response to increasing END concentration with AR transfected PC-3 cells exposed to DHT.

There have been relatively few studies on the endocrine specific effects of endosulfan mixtures or of the individual isomers. Our results demonstrate a previously unreported limited antagonist activity of END on both GR and ER in human cells. In a recent report using *in vitro* assays, technical grade endosulfan acted as an ER agonist and AR antagonist [305]. In some of my experiments significant antagonist activity for END on AR was observed but in most experiments no significant change was detected. When the data were combined, as shown in Figure 3-4A, no significant antagonism is present although there is a slight downward trend in luciferase response particularly at the highest concentration tested (10μM).

When END was tested for interaction with GR in PC-3 it was found to significantly antagonise the action of dexamethasone at 100 nM as determined by changes in firefly luciferase activity (Figure 3-4B). However for END concentrations of 1μM and higher the effects were not significant. When END alone was added to the transfected PC-3 cells it did not produce a change in luciferase response, suggesting it cannot function as a GR agonist in the concentration range tested.

Leblond *et al.* [306] reported an effect of endosulfan upon the secretion of cortisol in rainbow trout and Kannan *et al.* [307] reported an increase in T-cell apoptosis in response to endosulfan in accord with earlier reports of negative effects upon the immune system [308]. None of these reports detected any direct interaction of the endosulfan with GR and further work needs to be done in other *in vitro* and *in vivo* systems to verify my finding of possible GR antagonism.

Similarly, END was tested for estrogenic or antiestrogenic activity as described in materials and Methods and a significant decrease in firefly luciferase activity resulted from the addition of END at 1 nM and 1μM concentrations in the presence 17β-estradiol (Figure 3-4C). No effect was seen in the absence of 17β-estradiol.

In several studies with both *in vitro* and *in vivo* endpoints of estrogenic activity endosulfan has been characterised as a weak estrogen [309-311]. The weak antiestrogenic effects I have detected may not have been detected with these estrogen responsive endpoints at the low dose tested. Furthermore, a weak agonist may appear to be an antagonist at low concentrations if it binds stably to the receptor, but is a poor transcriptional activator. In addition, technical grade endosulfan was used of which the β-isomer, used in a pure form in my work, comprises only about 30% [286]. Therefore the dosed rats received less than 0.5 mg/kg/day, which may be below the required dose to observe endocrine effects.

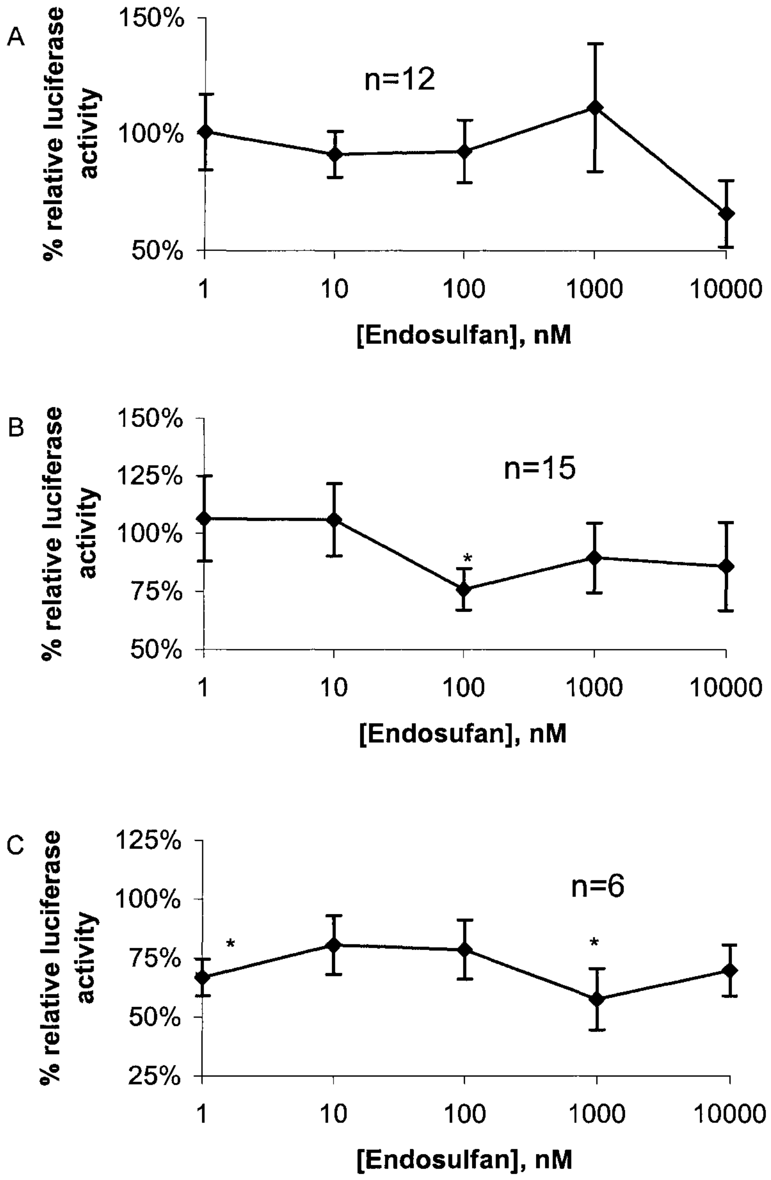


Figure 3-4: The effects of beta-endosulfan on receptor mediated luciferase transactivation. On AR with 1nM DHT in PC-3 cells (A); on GR with 1nM DEX in PC-3 cells (B); on ER with 1nM E2 in HepG2 cells (C). The * indicates value is significantly different ($p<0.05$, using Student's T-test) from control (no test compound added, set at 100% relative luciferase activity). Number of individual wells (n=), from multiple experiments, used to derive the graph is shown. Error bars indicate the standard error of the mean (SEM).

3.3 Dichlorodiphenyltrichloroethane and Related Compounds

In 1939 Paul Muller of Geigy Pharmaceuticals in Basel demonstrated the effectiveness of 1,1'-bis(p-chlorophenyl)-2,2,2-trichloroethane (*p,p*'-DDT) as an insecticide. The potency of this compound against the mosquitoes which carry malaria and the lice carrying typhoid, along with the ease and low cost of preparation and use, produced a revolution in public health management for which Muller was recognised in 1948 with the Nobel Prize for Medicine.

On January July 7, 1972, the Environmental Protection Agency in the United States placed severe restrictions upon the use of this family of pesticides. This action was taken in response to widespread concern about the adverse long-term health effects of DDT in wildlife and humans. Evidence of reproductive abnormalities amongst fish and bird species, particularly around the Great Lakes, combined with evidence of the environmental persistence of DDT and its metabolites forced the United States administration and many other governments to take action. Nevertheless, many developing countries still rely on DDT as their primary agent for the control of malaria mosquitoes and other public health threats. To date no equally cost effective alternative to DDT has been made available and long-term environmental hazards are outweighed by immediate public health concerns.

3.3.1 Environmental Fate

DDT adsorbs very strongly to soil and is subject to evaporation and photodegradation at the surface of soils. It does not leach appreciably to groundwater nor readily hydrolyse but may be subject to biodegradation in flooded soils or under anaerobic conditions (see Figure 3-5). If released to water it will adsorb very strongly to sediments and be subject to evaporation and photo-oxidation near the surface. Biodegradation may occur in sediments via either aerobic or anaerobic microbial pathways. If released to the air it will be subject to direct photodegradation and react with photochemically produced hydroxyl radicals. Wet and dry deposition both result in removal from the atmosphere. Half-life estimates for DDT are typically around 10-20 years [312].

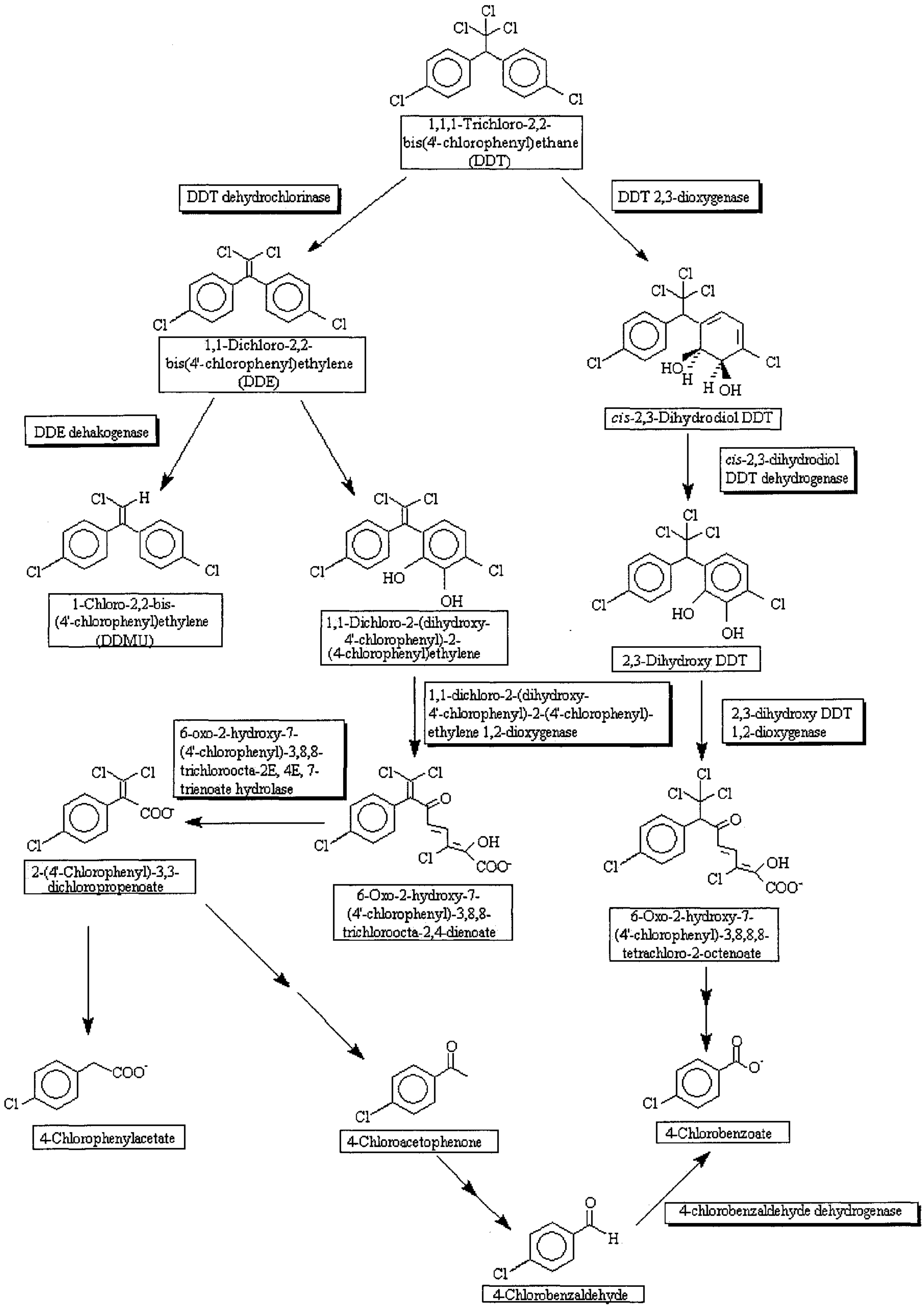


Figure 3-5: Typical biological degradation pathways for p,p'-DDT

Although *p,p*'-DDD is a major breakdown product of *p,p*'-DDT (Figure 3-5), it was released into the environment as an insecticide in its own right due to its better efficacy against certain insects. In addition it has found limited use as a chemotherapeutic for the treatment of adrenocortical carcinoma under the name Mitotane [313].

3.3.2 Human Exposure

Human exposure occurs mainly through ingestion of contaminated food, especially contaminated fish, dairy products and human milk. Estimated daily doses for American adults are in decline from a high of 240 μg/day in 1970, around 7 μg/day in 1979 to 1 μg/day in 1991 [312]. Intake in infants up to two years tends to be higher at around 3.5 μg/day but these levels are still below the WHO recommended maximum dose of 20 μg/kg/day. Amongst Canadian populations the highest exposures are amongst the Inuit whose traditional diet is rich in marine mammals which can carry levels of 300 μg/g wet weight or more [312].

3.3.3 Health Effects

The effects of DDT exposure have been studied across a range of species both in the laboratory and in the wild, including birds, reptiles, mammals and amphibians. Many toxic endpoints have been reported including effects on neurological and endocrine systems, development, reproductive success and even death [312]. It was the mounting evidence of catastrophic reproductive failure associated with high DDT levels in several bird species that in part prompted the United States government to restrict DDT use in the 1970s.

Acute exposure to DDT and/or its derivatives as experienced by agricultural and other pesticide workers produces hepatic and neurological effects. Chronic exposures have been implicated in an array of toxic outcomes including effects on the immune, reproductive and endocrine systems. In the liver there is considerable evidence for DDT causing an elevation in the expression and activity of several enzymes including alanine and aspartate aminotransferases and alkaline phosphatase as well as mixed function oxidases in both humans and experimental animals [314-316]. In rats CYP2B, 3A1 and 3A2 activities all increased with DDT dose, but CYP1A did not [317-319]. The persistent

DDT breakdown product *p,p'*-DDE was also found to increase P450 expression and produce an increase in liver weight in rats [319-321].

Acute neurological effects in humans of exposure to DDT include nausea, vomiting, headache and perspiration [322, 323]. In animal studies similar effects have been observed with effects as severe as partial paralysis in guinea pigs [324-329]. Investigation of the mechanisms underlying these effects has indicated that DDT and related compounds accumulate in the brain and may alter brain lipid composition and impact on neurotransmitter release [330-332]. Observations in wildlife undergoing starvation indicate that stress and dietary restriction exacerbate the neurotoxic effects of DDT [312, 333]. This may explain why the impact of DDT exposure in wildlife, where periodic starvation frequently occurs, for example during migration, can be particularly severe.

Several animal studies have indicated that chronic exposure to DDT may impair the immune response. Doses as low as 0.3 mg/kg/day given to mice for 16 weeks or more have been shown to affect both the humoral and cell mediated immune response to an antigen challenge [334-336]. As with most studies the authors did not examine the levels of metabolites in these animals so the active ingredient could well be a breakdown product such as DDE.

There have been numerous reports of effects of DDT exposure on reproduction in humans, experimental animals and wildlife. In humans there are several reports showing a link between premature birth or pregnancy failure and maternal DDT burden [337, 338]. In addition it has been suggested that DDT exposure can decrease duration of lactation in repeat breast feeders [339-341] from seven and a half months in women with less than 2.5 ppm in their serum to three months in women with serum concentrations of 212.5 ppm.

Similar results have been repeated in experimental animals and attempts have been made to better understand the underlying mechanisms. Several investigators have found effects upon the endocrine system including decreased progesterone and sterility in female rats [342] and more recent reports of anti-androgenic activity [343].

Research on various bird species has shown several common reproductive effects including decreased egg size, increased embryo lethality, delayed oviposition, and testicular effects including decreased testis weight and abnormal morphology [344-350].

Reduced levels of LH and E observed in conjunction with these effects may explain some of the outcomes [330, 351].

A range of reproductive and developmental effects has been attributed to maternal exposure to DDT across a variety of other species. Mice exposed perinatally to DDT via maternal dosing at 34.3 mg/kg/day exhibited increased neonatal mortality and neurological impairment [352]. Preweaning mortality increases were seen by other investigators using similar maternal doses. In addition, several of these groups and others observed premature puberty in the surviving offspring [353-356]. More recently reproductive and developmental effects in Florida alligators have been attributed to DDT and related compounds. Decreases in clutch viability, phallus size, serum testosterone levels and altered steroidogenesis were all observed in animals from a highly contaminated lake in comparison to those living in relatively clean watersheds [357-359]. These effects have been verified as being at least in part attributable to DDT by exposing alligators in a controlled setting where exposure to other contaminants is eliminated [360].

It is believed that many of these effects are the result of the interference of DDT and related compounds with the normal functioning of the endocrine system. Several reports have indicated disruption of the androgen [343, 358] and LH balance [330, 361, 362] as mentioned above. Other reports of effects in animals which suggest disruption of the endocrine system by DDT and related compounds include observations of production of the egg protein vitellogenin by male alligators and fish [363-366], suggesting exposure to a potent estrogen, and delayed tadpole metamorphosis in exposed amphibians, a process regulated by thyroid hormones [363-366].

3.3.4 Results

In light of the evidence for DDT and related compounds producing endocrine effects we decided to investigate the effects of DDT and several of its related compounds in our cell culture steroid hormone assay system. When the effect of *p,p*'-DDT on steroid receptor mediated luciferase transactivation was measured in cell culture with AR, GR and ER it was found that only with AR was a statistically significant change observed at one concentration (Figure 3-6). This compound antagonises the action of 1 nM DHT on AR. No effect was seen in the absence of known ligand for any of the receptor types (Figure 3-6).

In a similar investigation into the effects of *o,p*'-DDT on steroid receptor mediated luciferase transcriptional regulation this isomer of DDT was able to change the activity of all three receptors (Figure 3-7). On AR there appears to be an antagonist effect over the concentration range tested with luciferase levels reduced by almost 70% at 10μM. With both GR and ER there is an apparent agonist response at only one concentration and only in the presence of known ligand, dexamethasone or estradiol, respectively. This agonsitic action is seen at concentrations as low as 1 nM with GR, but is only seen with ER when *o,p*'-DDT is applied at above 1μM.

The DDT metabolite *p,p*'-DDE exhibited a significant antagonist trend on AR, reducing DHT induced luciferase activity by 60% in cells exposed to the compound at 1μM or higher concentrations (Figure 3-8). GR showed no response either in the presence or absence of dexamethasone. With ER a mixed response was observed with *p,p*'-DDE whereby significant antagonism at low concentrations which reduced luciferase activity by almost 50% at 10 nM disappeared at the highest concentration tested, 10μM. The large error bars on this last value make it hard to determine the significance of this increase.

When *p,p*'-DDD was added to transfected cells expressing steroid receptor induced luciferase it produced no significant effect with AR (Figure 3-9). No significant effect upon luciferase expression either with or without DEX was seen for GR although there was a downward trend above 1μM concentration. When *p,p*'-DDD was used to treat HepG2 cells transfected with the ER at 10 μM (the highest concentration tested) there was a significant antagonist effect versus 1nM E2.

3.3.5 Discussion

Despite the structural similarity of this family of compounds it is of interest to note that there was no universal response seen with any of the three steroid receptors tested. AR response was affected by three of the four compounds with both *o,p*'- and *p,p*'-DDT as well as *p,p*'-DDE producing an antagonist response atone or more concentrations. The least potent of the antagonist effects was produced by *p,p*'-DDT while *o,p*'-DDT produced the most potent effect causing 50% decrease in luciferase activity at 100 nM. This pattern correlates well with the published literature with regards to the activities of these compounds [188, 198, 199, 367]. Both DDT isomers and *p,p*'-DDE have been shown to be mammalian AR antagonists [198, 202, 368]. Furthermore there are reports of both DDT isomers binding to a teleost AR with relatively high affinity [369]. The AR2 from Atlantic Croaker (*Micropogonias undulatus*) has not been sequenced but other teleost AR sequences share substantial homology with human AR particularly in the ligand-binding domain. For example the C-terminal portion of goldfish AR (gi20135660) is 68% identical to that of human (gi4557331).

In contrast, GR was the least responsive of the receptors to this family of compounds with significant effects produced only by treatment with *o,p*'-DDT which acted as an agonist in the presence of DEX but had no effects at the concentrations tested alone. To date none of the DDT family has been shown to interact directly with GR. One report describes a synergistic activity between glucocorticoids and DDT in the induction of the liver enzyme gamma-glutamyltranspeptidase [370]. Although this group used a technical grade of DDT which contains a mixture of *o,p*'- and *p,p*'- isoforms it would still seem to corroborate our findings.

Estrogen receptor exhibited a response with both *o,p*'-DDT and *p,p*'-DDE. Luciferase activity in the cell extracts was increased by the former at concentrations of 10μM or above and decreased by the latter even at the lowest concentration tested (1 nM). Numerous investigators have reported estrogenic activity for *o,p*'-DDT [38, 367, 371] so the reproduction of this result in our assay serves to further validate our methodology. Evidence for *p,p*'-DDE interacting with ER is limited. Matthews at al. [371] found that *p,p*'-DDE bound to recombinant ERs from several species including human, in contrast Chen *et al.* [367] found that *o,p*'-DDE but not *p,p*'-DDE bound and activated gene expression through ERα in a yeast based assay. The difference in co-factor milieu between cell free, yeast and human cells may account for this apparent discrepancy.

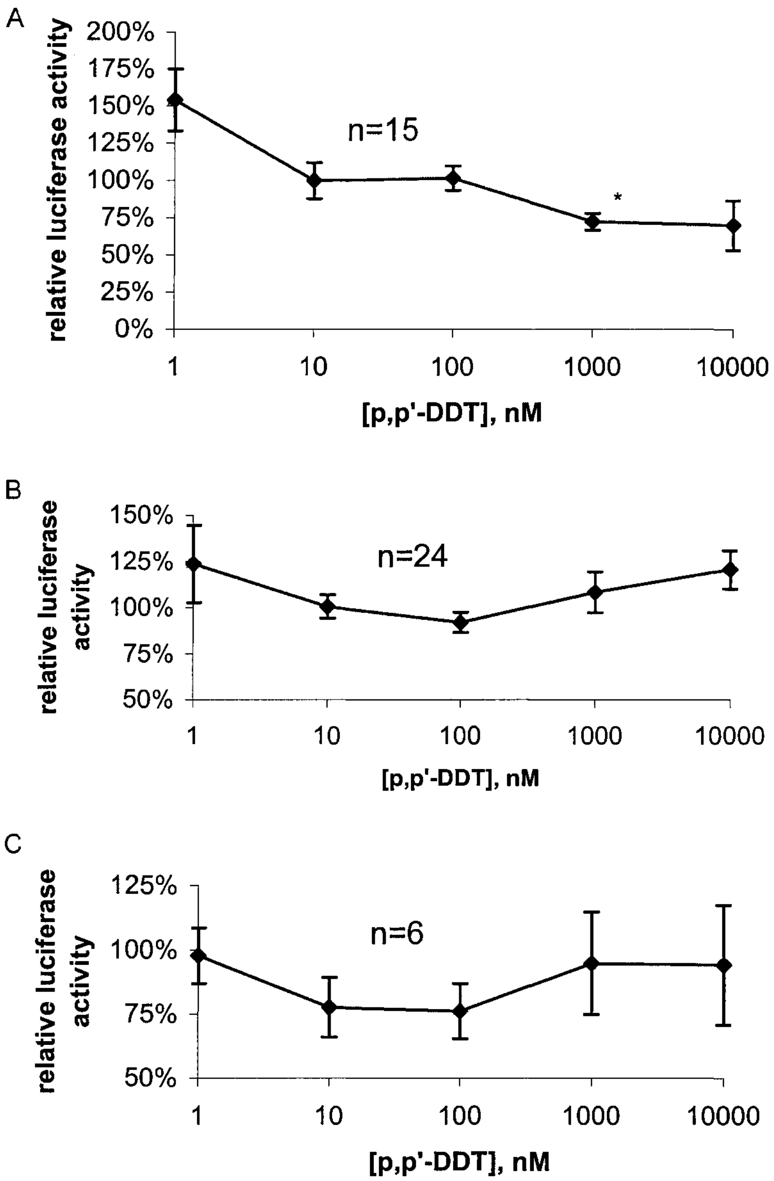


Figure 3-6: The effects of *p,p'*-DDT on receptor mediated luciferase transactivation. On AR with 1nM DHT in PC-3 cells (A); on GR with 1nM DEX in PC-3 cells (B); on ER with 1nM E2 in HepG2 cells (C). The * indicates value is significantly different ($p<0.05$, using Student's T-test) from control (no test compound added, set at 100% relative luciferase activity) . Number of individual wells (n=), from multiple experiments, used to derive the graph is shown. Error bars indicate the standard error of the mean (SEM).

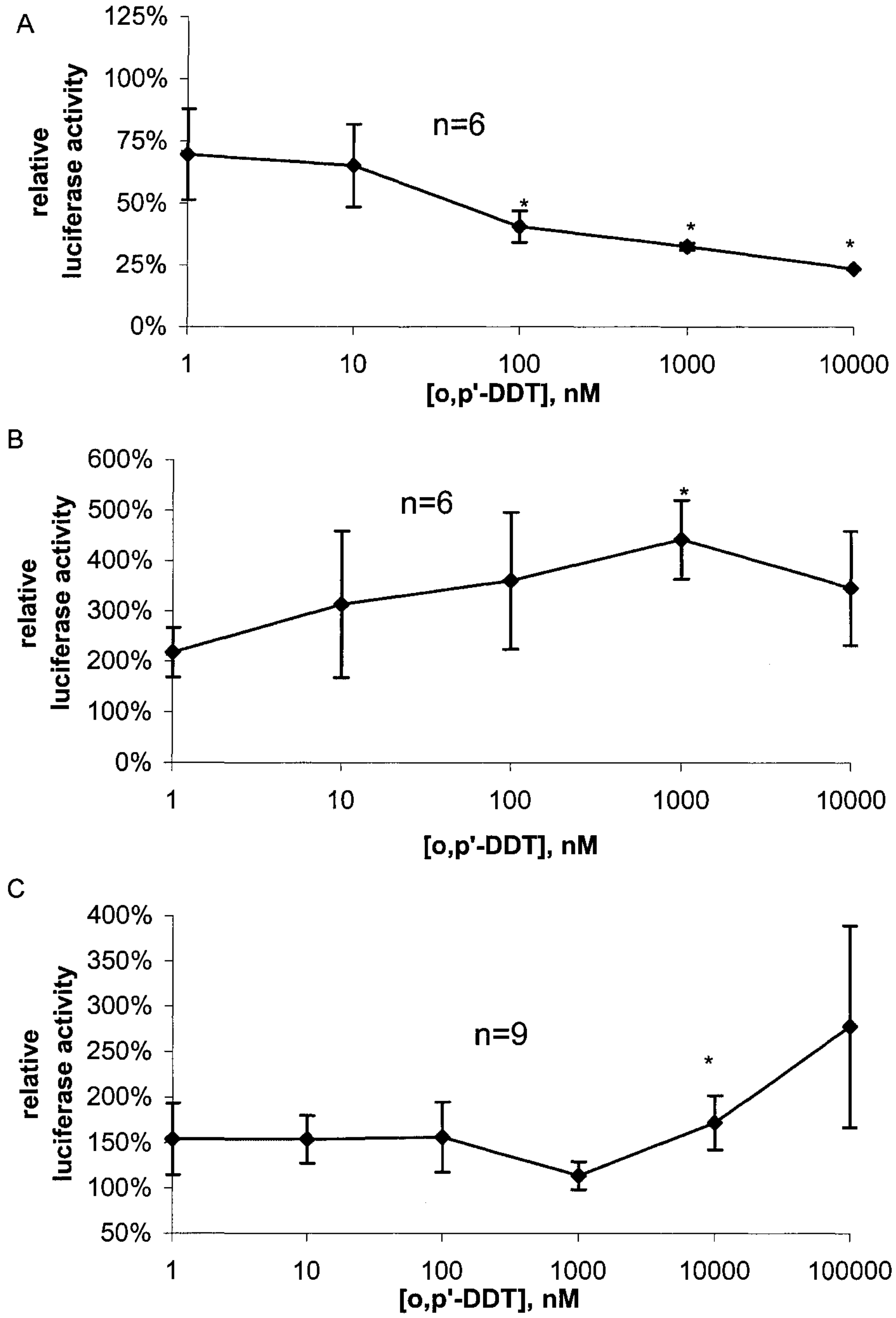


Figure 3-7: The effects of *o,p'*-DDT on receptor mediated luciferase transactivation. On AR with 1nM DHT in PC-3 cells (A); on GR with 1nM DEX in PC-3 cells (B); on ER with 1nM E2 i HepG2 cells (C). The * indicates value is significantly different ($p<0.05$, using Student's T-test from control (no test compound added, set at 100% relative luciferase activity) . Number of individual wells (n=), from multiple experiments, used to derive the graph is shown. Error bar indicate the standard error of the mean (SEM).

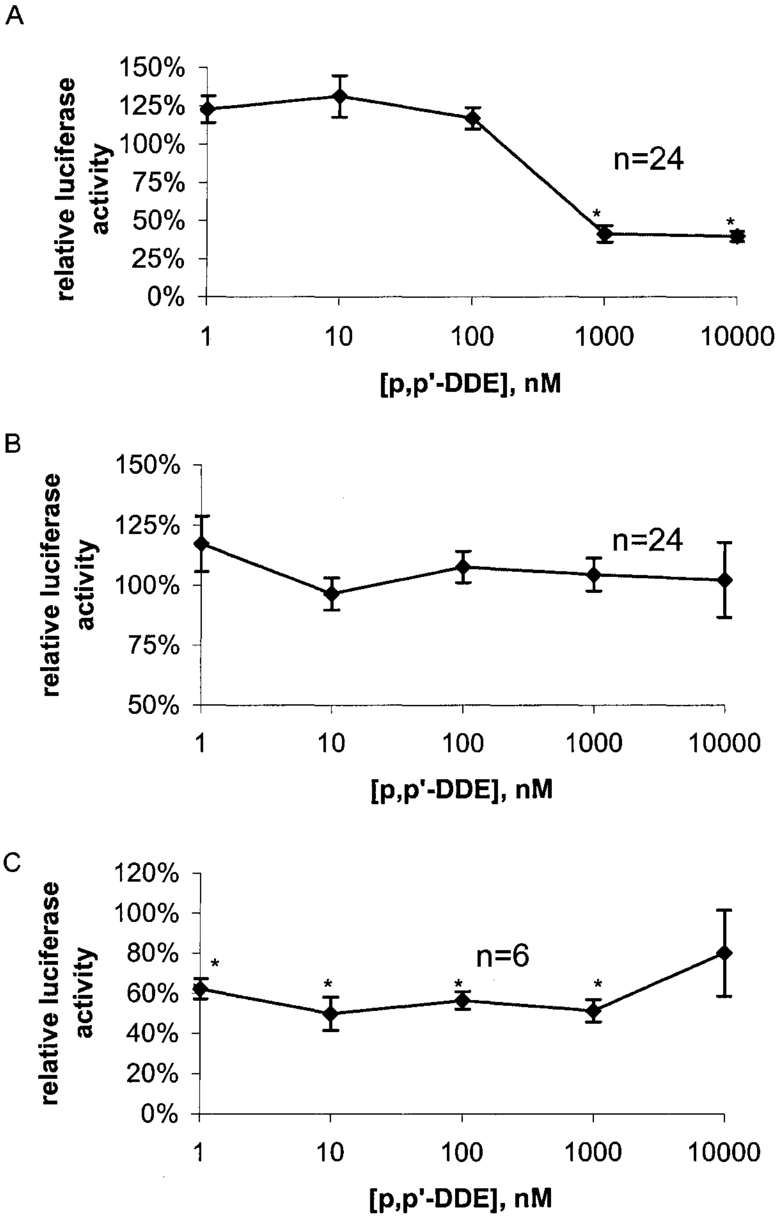


Figure 3-8: The effects of *p,p'*-DDE on receptor mediated luciferase transactivation. On AR with 1nM DHT in PC-3 cells (A); on GR with 1nM DEX in PC-3 cells (B); on ER with 1nM E in HepG2 cells (C). The * indicates value is significantly different ($p<0.05$, using Student's T-test) from control (no test compound added, set at 100% relative luciferase activity) . Number of individual wells (n=), from multiple experiments, used to derive the graph is shown. Error bars indicate the standard error of the mean (SEM).

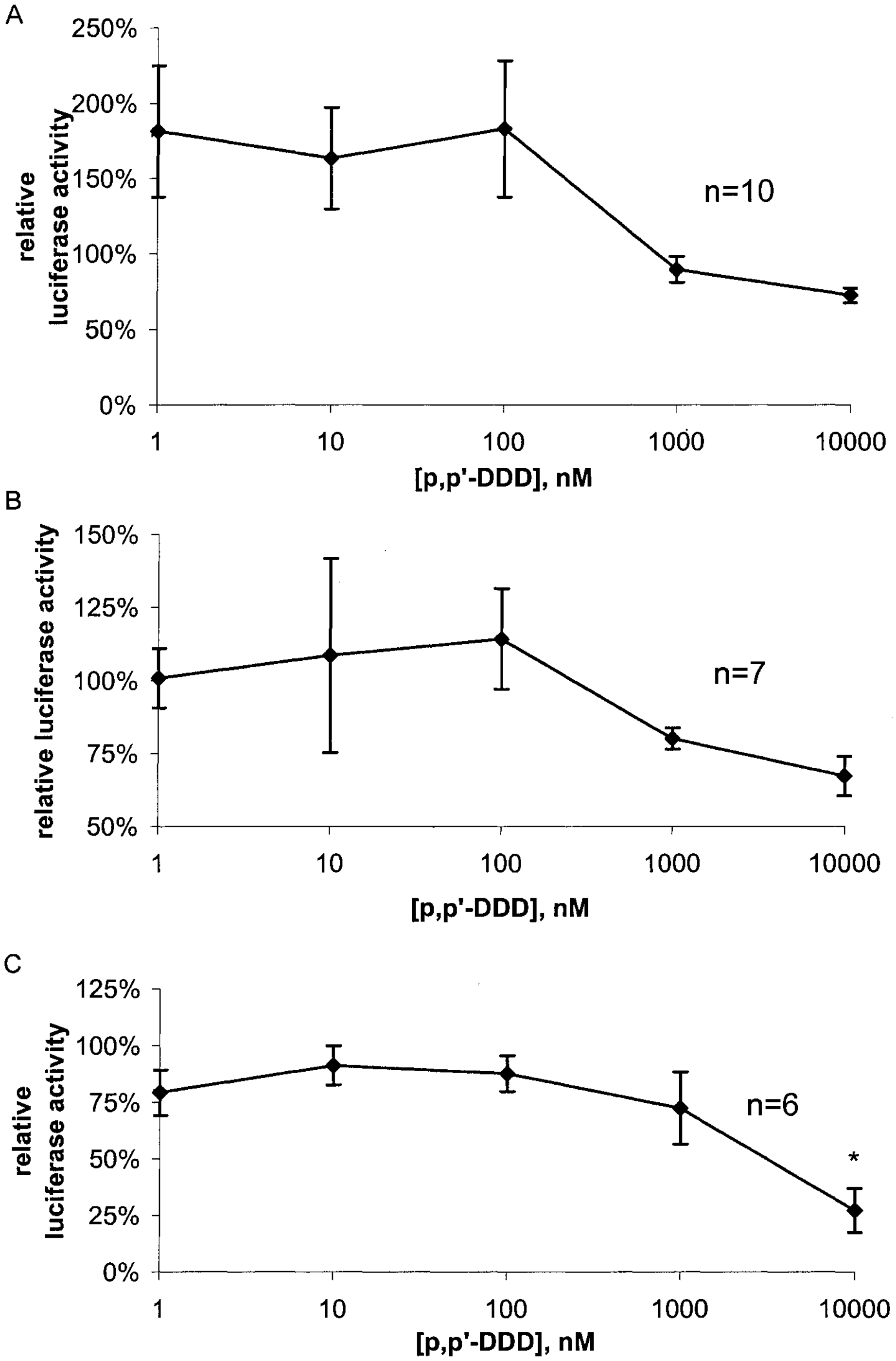


Figure 3-9: The effects of *p,p'*-DDD on receptor mediated luciferase transactivation. On AR with 1nM DHT in PC-3 cells (A); on GR with 1nM DEX in PC-3 cells (B); on ER with 1nM E2 in HepG2 cells (C). The * indicates value is significantly different ($p<0.05$, using Student's T-test) from control (no test compound added, set at 100% relative luciferase activity) . Number of individual wells (n=), from multiple experiments, used to derive the graph is shown. Error bars indicate the standard error of the mean (SEM).

3.4 Pulp and Paper Mill Effluents (PPME)

Various by-products of the wood pulping process are released during the multiple steps involved in converting wood into paper. Several of these effluents have caused concern over the years as various toxins have been identified within them, perhaps most notably PCDDs released by chlorine bleaching processes. To protect human health and the environment, alternative methodologies have increasingly been adopted and effluents are now frequently retained for some form of processing prior to release into the environment, typically a river, lake or the ocean. The effluents comprise a huge array of compounds, including both natural ones produced by the plant and many synthetics produced by either reactions of a natural product during the processing or residues and products of the chemicals added. The half-life of these compounds is equally variable and the various components of any one effluent can encounter very diverse fates. Some, particularly organochlorines such as PCDDs and polychlorinated dibenzofurans, can be extremely persistent but many of the natural components are readily broken down.

3.4.1 Health Effects

Amongst pulp mill workers most of the health effects reported are attributable directly to the chemicals they work with or the physical environment of the plant. For example there are several reports of elevated pleural and lung cancer rates [372-374] but these are proposed to result from exposure to asbestos and other dusts present in paper mills. Recently Band *et al.* [375] reported that in a large study of pulp mill workers in British Columbia there was an elevated incidence of prostate, stomach and rectal cancers and leukaemia particularly amongst those employed in the industry for more than 15 years. An increased incidence of leukaemia and gastrointestinal malignancy had been previously associated with pulp and paper mill workers in several previous studies [376-379]. In contrast, the link to prostate cancer and its associated endocrine basis has only been reported by one other group [380, 381].

Studies in a variety of vertebrates support the possibility of an endocrine mechanism for toxicity resulting from exposure to pulp mill effluent. Several investigators have reported androgenic or estrogenic activity associated with pulp manufacturing [382-384]. This seems to result in the depression of serum hormone levels and masculinisation of females in some fish species [385, 386]. In addition PCDDs produced by bleaching methods historically employed by many pulp mills interact with the AhR which can result in an antiestrogenic activity [160, 172, 173, 387]. Although modernisation of most facilities in recent years has reduced emissions of PCDDs and other halogenated organics dramatically, emissions remain a health and environmental issue due to their resistance to degradation.

Apart from acute exposure of pulp mill workers, the major route of exposure to effluents is through drinking water and for more stable components of the mixtures, such as PCDDs, via bioaccumulation in the food web particularly in fish. Due to the diversity of compounds produced it is not possible to give exposure figures for pulp mill effluent. Clearly for those who draw their water supply and fish from waterways downstream of pulp and paper industry exposure will be greater than those upstream.

3.4.2 Results and Discussion

Black liquor is the product of the initial lignin removal step performed upon crushed wood chips. Typically the wood is cooked in sulphite or sulphate (Kraft process) solution under pressure and black liquor represents the resultant liquid effluent fraction. For the purpose of these experiments black liquor was obtained from three different pulp mills, two in British Columbia and one in Ontario. This liquor was diluted into DMSO and added at 1% or below of the final concentration to buffered media which was then applied to transfected PC-3 cells in the AR transactivation assay as described in Materials and Methods.

Both Harmac and Elk Falls samples appear to contain androgenic activity, since in all cases an upward trend is seen in the presence of 0.75 nM DHT, however only the results for Harmac were statistically significant ($p<0.05$; Figure 3-10). A finding of androgenic activity in black liquor correlates well with the literature reports of androgenic activity in pulp mill effluents [382, 383, 385, 386]. Due to the low pH and apparent presence of cytotoxic compounds within the extract significant cell death occurred when concentrations above 0.1% were applied to the cells.

Since the black liquor represents a chemically derived tree extract, it is not clear whether this activity stems from a natural extract of the wood or is a synthetic bi-product of the pulp making process. In our investigation of the androgenic activity of white liquor (the recycled sulphate/sulphite solution applied to the wood chips) no androgenic or antiandrogenic activity was detected (data not shown) indicating that the activity is a product of the wood chip extraction process. I performed the androgen assay on five other fractions from one of the pulp mills but none yielded consistent androgenic or antiandrogenic activity (data not shown).

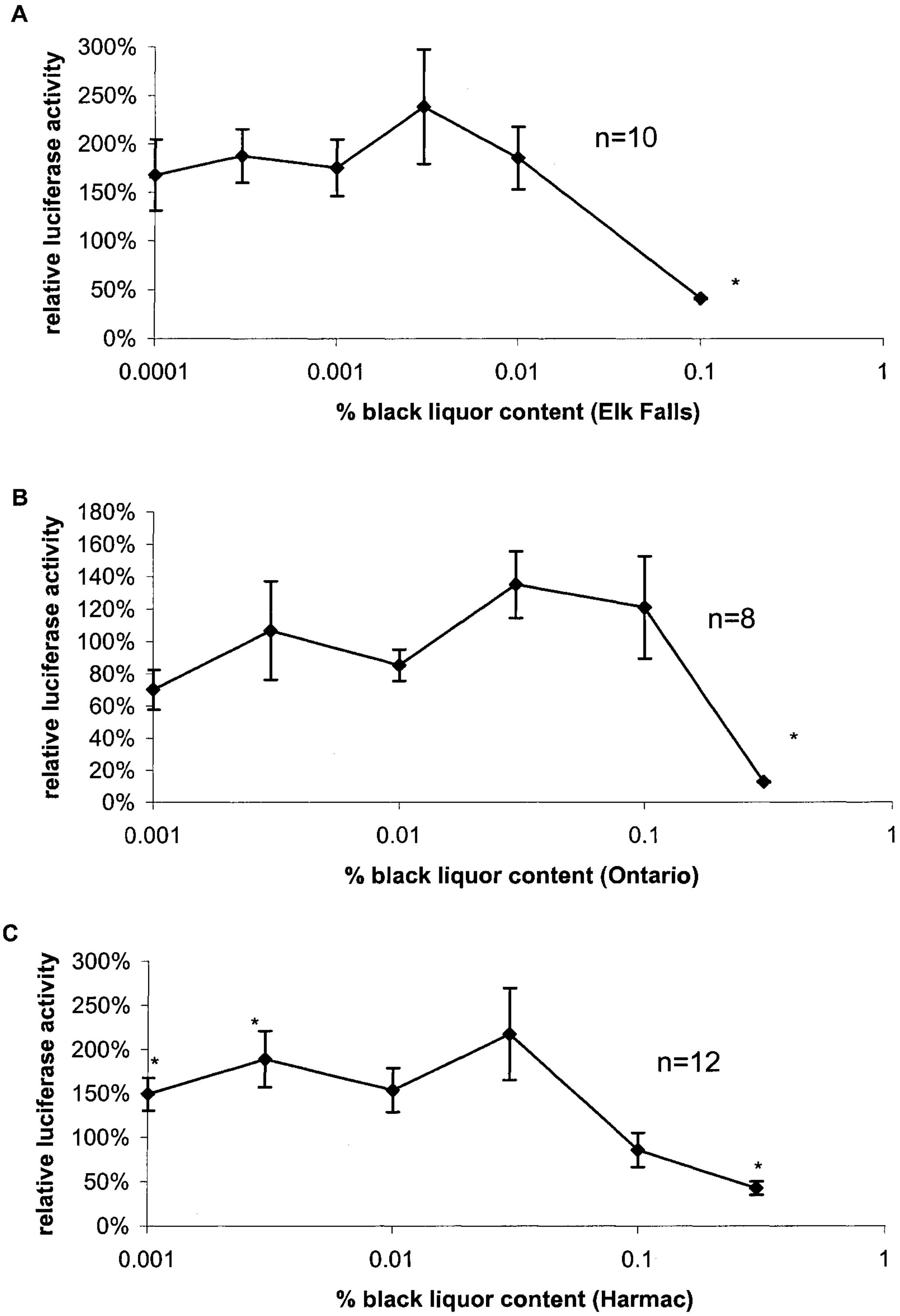


Figure 3-10 The effects of black liquor on androgen driven transactivation in PC-3 cells. Black liquor from three different pulp mills was serially diluted in DMSO and applied to transfected PC-3 cells in media containing 0.75nM DHT. Luciferase activities measured from repeat assays were combined and presented as percent fold activation.

The * indicates value is significantly different ($p<0.05$, using Student's T-test) from control (no black liquor added, set at 100% relative luciferase activity). Number of individual wells (n=), from multiple experiments, used to derive the graph is shown. Error bars indicate the standard error of the mean (SEM).

3.5 Fraser River Sediment Extracts

As part of Environment Canada's ongoing Fraser River monitoring programme, sediment samples are collected on a regular basis from various sites, and from relatively clean reference sites within the Fraser River watershed. Sediment extracts were assayed by the methods described for the individual compounds tested above, and the results obtained were analysed and interpreted in the same manner. Table 3-1 summarises the effects of the sediment extracts on the three receptor systems.

3.5.1 Results and Discussion

Based on consistent results derived in two separate sets of experiments, endocrine activity was detected in 8 of the 11 sediment extracts tested on the androgen, estrogen and glucocorticoid receptors (Table 3-1). In the AR receptor system, sediments 5 and 16 produced an agonistic and antagonistic response, respectively (Figure 3-11A). In the GR receptor system, three sediments (3, 4 and 9) produced a detectable net change in luciferase expression in combination with the synthetic agonist, dexamethasone (Figure 3-11B). Sediment 3 produced an additive agonist response in combination with DEX, while sediments 4 and 9 produced a mixed response with an additive effect with DEX at concentrations below 0.1% sediment extract but this response declining such that sediment 4 acts as an antagonist of the DEX induced luciferase expression at 1 to 10%, and sediment 9 returns to control values. This effect may be the product of multiple compounds within the sediment extract acting upon the transactivation pathway. For sedimentTests with the estrogen receptor system showed an antagonist response in sediments 5,6,7 and 9 and an agonist response in sediment 15 (Figure 3-11C). None of the sediment extracts produced a detectable response without the addition of known agonist on any of the receptor systems.

In the sediment samples analysed by the AXYS Analytical Laboratories, the levels of most of the individual contaminants were in the pM to nM range per g of sediment. (Refer to the Appendix for concentrations in individual sediment samples.) Most of the effects that we observed with pure compounds were seen only when the test compound was applied to the cells at μM concentrations. It is therefore quite surprising that activity was detected in the sediment extracts. This may indicate the presence of novel potential

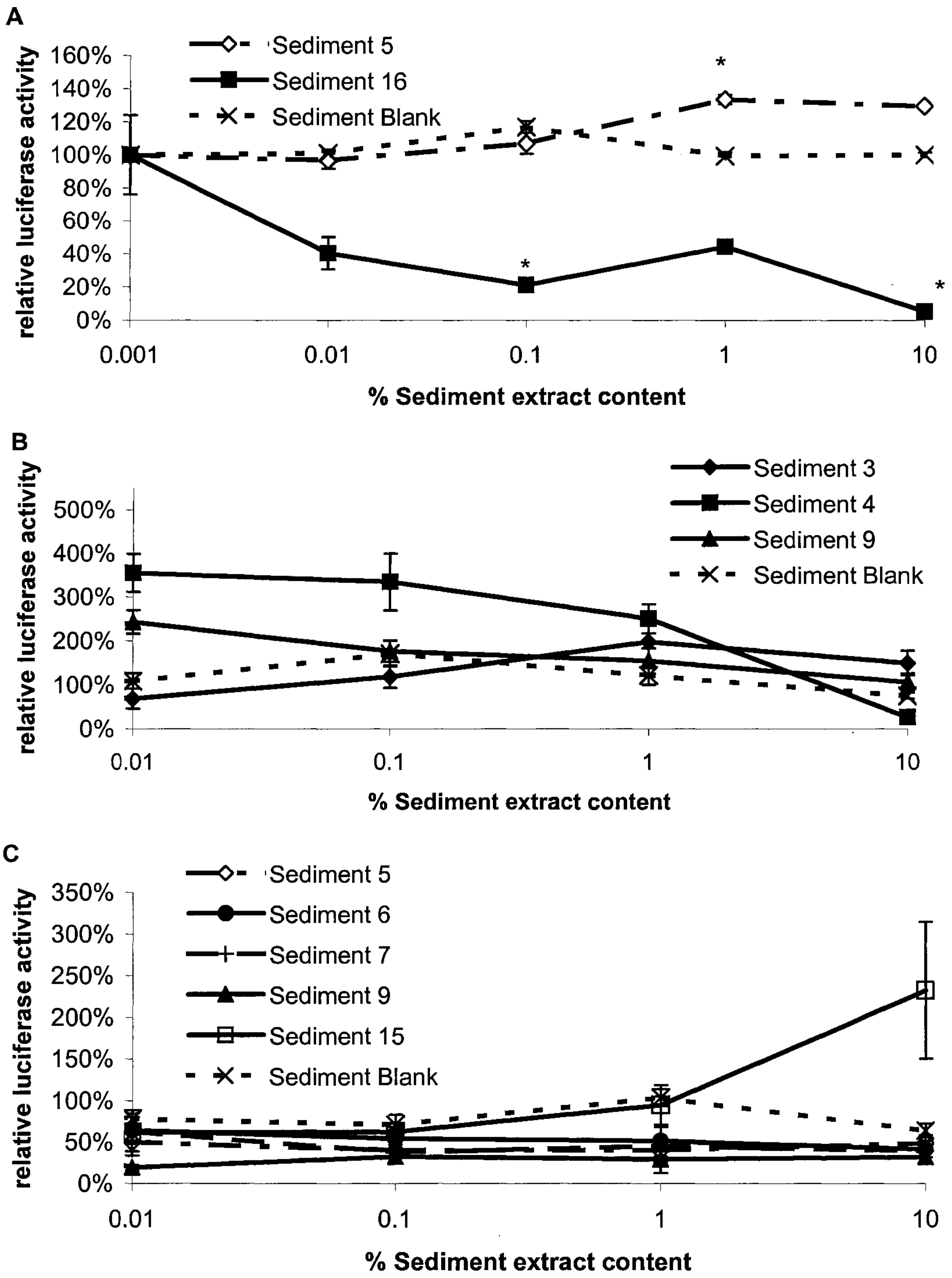


Figure 3-11: The impact of Fraser River sediment extracts on receptor mediated luciferase transactivation. Fraser River sediment extracts prepared by Environment Canada were applied to transfected cells in culture. Luciferase activities measured from repeat assays were combined and presented as percent fold activation. On AR with 1nM DHT in PC-3 cells (A); on GR with 1nM DEX in PC-3 cells (B); on ER with 1nM E2 in HepG2 cells (C). The * indicates value is significantly different ($p<0.05$, using Studen't T-test) from control (no test compound added, set at 100% relative luciferase activity) . Six individual wells, three from each of two experiments were used to derive each data point. Error bars indicate the standard error of the mean (SEM).

endocrine-disrupting chemicals or some that are not included in the analysis protocol (e.g. vinclozolin). Alternatively, there could be a synergistic effect produced by a combination of environmental contaminants. To investigate the potential for synergy I applied each of the pesticides and sediment extracts studied in combination with 1μM 4-nonylphenol, however I saw no synergistic effect. Although our observations are in agreement with a number of recent reports in which no synergy was observed, it does not eliminate the possibility of synergy under different conditions.

Test sample	**Response**	**Lowest dilution for response**	**Highest % change in RLU (conc.)**
Androgen receptor			
Sediment 5	agonist	0.1 x	33% (1 x)
Sediment 16	antagonist	0.01 x	-67% (1 x)
Estrogen Receptor			
Sediment 5	antagonist	0.01 x	-42% (0.1 x)
Sediment 6	antagonist	0.01 x	-52% (1 x)
Sediment 7	antagonist	0.01 x	-70% (1 x)
Sediment 9	antagonist	0.01 x	-66% (1 x)
Sediment 15	agonist	1 x	124% (10x)
Glucocorticoid Receptor			
Sediment 3	agonist	0.1 x	86% (1 x)
Sediment 4	agonist	0.01 x	196% (1 x)
Sediment 9	antagonist	0.1 x	-40% (1 x)

Table 3-1: Summary of interactions of sediment extracts with each receptor system
The % change in relative luminescence unit value is given as the maximal response level relative to the control. Sediments that are not included in the table elicited no response in the assay system.

The experimental outcome for each sediment sample was compared to the predicted outcome based upon the mixture of contaminant compounds identified in the chemical analysis (Table 3-1). In doing so it was assumed that the sediment extracts assayed have the same composition as the sediment analysed by AXYS Analytical. This assumption may be incorrect since the DMSO-soluble fraction used in the bioassays was obtained through a "raw" extraction, as opposed to the more refined methods employed by

AXYS Analytical which involved extensive cleanup procedures following the initial extraction. As a result the absolute levels of some components will vary between the GC-MS analysed and my cell culture tested extracts.

Sediment extracts 1, 2, 3 and 4 correspond to reference sites in the Fraser River. Of these, only Sediment 1 contained any detectable contaminants (PCBs or 4-NPs). The level of 4-NP in this sediment is around 50 nanomolar, which, in pure form, was sufficient to give a weak agonist response in our assays with the androgen, glucocorticoid and estrogen receptors. However, sediment extract 1 gave no response when tested on any of the three receptor systems. Since neither NPs nor PCBs are present in sediment extracts 2-4, it might be expected that no response would be seen for these extracts with any of the receptor assays. However this did not prove to be the case. Both sediments 3 and 4 gave a response with the glucocorticoid receptor system. Constituents of the sediment extracts for which the endocrine potential has not been assessed, and thus cannot be accounted for, may explain these apparently anomalous results. The additional contribution of these compounds could be responsible for eliciting the unexpected outcomes.

#	**AR**		**ER**		**GR**	
Outcome	*Observed*	*Predicted*	*Observed*	*Predicted*	*Observed*	*Predicted*
1	no effect	NP -	no effect	NP +	no effect	NP 0
2	no effect	none	no effect	none	no effect	none
3	no effect	none	no effect	none	agonist	none
4	no effect	none	no effect	none	agonist	none
5	agonist	NP -	antagonist	NP +	no effect	NP 0
6	no effect	NP -	antagonist	NP +	no effect	NP 0
7	no effect	NP - PCBs -	antagonist	NP + PCBs 0	no effect	NP 0 PCBs -
8	no effect	NP -	no effect	NP +	no effect	NP 0
9	no effect	NP - PCBs -	antagonist	NP + PCBs 0	antagonist	NP 0 PCBs -
15	no effect	NP - PCBs -	agonist	NP + PCBs 0	no effect	NP 0 PCBs -
16	antagonist	NP - PCBs -	no effect	NP + PCBs 0	no effect	NP 0 PCBs -
Blank	no effect	none	no effect	none	no effect	none

Table 3-2: Observed and predicted responses of receptor assays to sediment extracts. The Predicted Effect column indicates compounds detected in the sediment extracts from GC-MS analysis performed by Axys Analytical and their predicted effects. NP is 4-nonylphenol, predicted responses are: + agonist; - antagonist; 0 no effect.

Sediment samples for extracts numbered 5, 6, 7 and 8 were taken from the Main Arm of the Fraser River, and samples for extracts numbered 9, 15 and 16 were derived from the North Arm. In addition, a blank was prepared in which the extraction procedure was followed in the absence of sediment. For most of these sediment samples, an effect on luciferase expression was seen with at least one receptor system. However, in only about half of the cases did the observed outcome correlate with the predicted effect by extrapolation of the activity of the detected target compounds in the sediment extracts. This may indicate that there are one or more unidentified contaminants that are widely distributed in the Fraser River sediments that are able to interact with these steroid receptor assays.

In our testing with pure compounds, the pesticides (HEX, END, or DDT) were only found to possess endocrine activity when present in the micro to nanomolar range. Since these compounds are present in the sediments at much lower concentrations than this (in the pico- to femtomolar range), it is unlikely that these compounds contribute to the net endocrine activity seen in some sediment extracts. This does not indicate that these compounds are not environmentally important, since bioaccumulation of these compounds through the food chain or prolonged exposure may cause the levels in individual tissues and organisms to be considerably higher than those found in the sediments. The 4-nonylphenols and PCBs are present in the sediments at nanomolar concentrations, which is close to the values at which endocrine activity was observed in our assays (see chapters 4 and 5 respectively). It seems reasonable to assume, therefore, that these compounds are able to contribute to the total endocrine activity of the sediment extracts. It also seems likely that there are other compounds present in the mixtures that are able to contribute to the net endocrine activity of the sediment extracts. These compounds may be amongst those detected by GC-MS but not tested in pure form in our assay, or may represent known or unknown endocrine active compounds not measured by the GC-MS analysis. Although the solvent was controlled for direct agonistic activity, it is possible that DMSO itself somehow facilitates the activity of ligands for the steroid hormone receptors, perhaps by altering the permeability of the cell membrane to hormones.

3.6 Closing Comment

In this analysis attempts to predict the endocrine activity of an environmentally derived mixture such as the sediment extracts or the pulp and paper mill fractions from knowledge of the activity of some of the components has proven to be of limited value. Additional activity can come from two sources: compounds that are detected but have not been tested for their endocrine potential, and compounds that were not measured, or are below detection thresholds in the original analysis. An additional impediment to this approach is the possibility of combinatorial responses from multiple concurrent exposures. Although early reports of synergy between environmental contaminants proved unreproducible and have been set aside, the possibility has not been ruled out since given the multifaceted nature of transcriptional regulation it seems possible that factors acting upon different aspects could produce greater than additive overall effect. Indeed since it is already known that the endocrine system is not a collection of autonomous pathways but an integrated whole, simultaneous impairment at multiple points in that network could produce an effect greater than the sum of the individual activities. For example, the combination of an estrogenic action, and an antiandrogen together may achieve greater physiological impact than either alone. This hypothesis seems worthy of future investigation, and could be readily if not exhaustively tested using the collection of endocrine active compounds identified here.

Chapter IV - Alkylphenols

Alkylphenols (APs) are both the precursors and the breakdown products of a family of industrial products used primarily as surfactants and in the manufacture of plastics. Nonylphenol (NP) is the most commercially prevalent of the alkylphenol family, representing approximately 85% of the alkylphenol market with octylphenol (OP) representing about 10%. APs are rarely used "as is" but are typically modified to produce alkylphenol polyethoxylates (APEs), tris (nonylphenyl) phosphite (TNPP) and nonylphenol-formaldehyde condensation resins (Figure 4-1).

APEs comprise the third largest group of domestic and industrial surfactants in use worldwide, with annual production of 600,000 metric tons per year. APEs are important in a number of industrial applications, including: as detergents in pulp and paper making and textile manufacturing; and as an ingredient in coatings, dispersants for agricultural pesticides, lubricating oils and fuels, and plastics manufacture. Such industrial applications comprise 55% of the total APE market. The remaining market is comprised of industrial and institutional cleaning products (30%), and household cleaning products (15%). APEs are also used in specialized applications including as intestinal permeability enhancers in drug delivery systems and in spermicides and contraceptive foams.

Releases of alkylphenols to the environment originate from a variety of sources including industrial outflows (particularly textile manufacturing facilities), vehicle washing, agricultural operations, and urban runoff either directly or via municipal sewage treatment plants. It is estimated that over 37% of the total APEs used are discharged directly to aquatic environment. Analysis of drinking water in New Jersey, USA, identified the presence of ten nonylphenol ethoxylates (NPEs) and one octylphenol ethoxylate (OPE) each at concentrations of 15-29 ng/L [388]. Levels of NP and the lower ethoxylates in ambient waters and sediments have also been studied. In US rivers NP concentrations in ambient water were found to be as high as 0.64 ppb and NPE up to 15 ppb, while in sediment NP was found at concentrations up to 2.96 ppm [389].

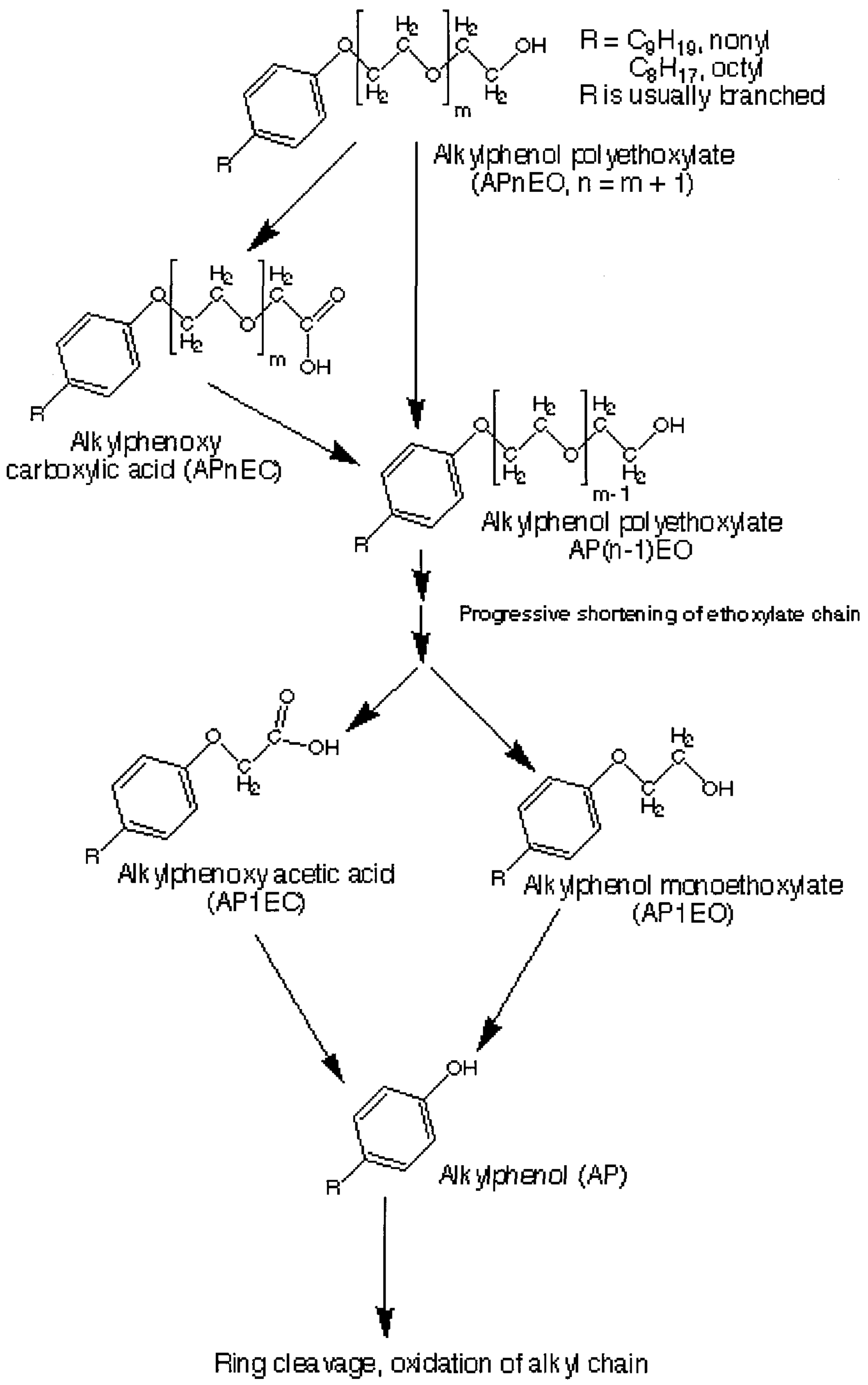


Figure 4-1: Structures of alkylphenol ethoxylates and their degradation to alkylphenol in the environment.

The ethoxylate groups are readily removed by microbial culture, such as those found in sewage treatments facilities and in freshwater sources. The AP core itself appears to be relatively impervious to environmental breakdown (Figure 4-1). The lipophilicity of APs promotes sedimentation in aquatic environments and accumulation in biota. Due to the array of applications to which the alkylphenols and their ethoxylates have been put, human exposure occurs through many and varied routes. Examples include: NP leaching into intravenous fluids [390]; NP leaching into milk from PVC tubing used in milk plants [391]; NP leaching into food from food-grade PVC used in packaging [392, 393]; spermicides containing octoxynol or nonoxynol as active ingredients; personal products (e.g. lotions, hair colourings, shaving cream, deodorant); and drinking water, at approximately 1 ppb (total nonylphenolics), as measured in both New Jersey [388] and Barcelona, Spain [394]. At least one report has demonstrated that NPEs are identifiable in the urine of non-exposed human control subjects, as well as exposed subjects, in a study of detergent-augmented chemotherapy [395]. Alkylphenols have also been detected in human umbilical cords [396] and breast milk [397].

4.1 Health Effects

4.1.1 Toxicity of Alkylphenolic Compounds

Although alkylphenol contamination usually enters the environment in the form of ethoxylate products, studies in various organisms, particularly fish and aquatic organisms, have shown that the breakdown products are generally more toxic than the original compounds [398]. Historically, toxicity testing methods indicated that alkylphenols posed no environmental or health threat at levels being released to the environment but these tests were not sensitive to subtle estrogenic or other hormonal effects. Currently there is still active debate in many countries about what is a safe level for alkylphenols in the environment.

4.1.2 Estrogenic Effects of Alkylphenolic Compounds

The first evidence that alkylphenols may be estrogenic came in the 1930s [399] where 4-propylphenol fed to ovariectomized rats partially restored estrogen function. Further evidence, published forty years later [400], demonstrated that several alkylphenols could compete with estradiol for binding to its cellular receptor. The discovery in 1991 that nonylphenol leaching from plastic culture vessels was able to stimulate proliferation in MCF-7 human breast cells in culture led to widespread health concerns [401]. This and more recent research [402, 403] has shown that the growth of these cells is increased by alkylphenols at concentrations one to ten thousand fold higher than the estradiol levels required to produce the same growth. Estrogenic effects have also been demonstrated in rainbow trout hepatocytes and chicken embryo fibroblasts [404, 405]. Furthermore, a recombinant yeast screen using the human estrogen receptor has shown similar results [406] and binding experiments with purified estrogen receptors α and β indicate that APs can directly bind to and activate them [404, 405].

The testes of fetuses from pregnant rats dosed with 4-octylphenol or DES were examined on day 17 after fertilisation. The activity and mRNA levels of cytochrome P450 XVII (17-α-hydroxylase; one of the enzymes involved in the synthesis of testosterone) was significantly reduced in fetuses exposed to 600 mg/kg of 4-octylphenol, or DES at 100 or 500 μg/kg, when compared with controls [407]. These results provide one mechanism by which estrogenic chemicals can affect fetal steroid synthesis and male development.

There is some evidence that alkylphenolic compounds can accumulate in organisms exposed to them [408, 409]. The bioconcentration factors calculated combined with current environmental levels of alkylphenolic compounds would mean that in some organisms body burdens would reach estrogenic effect levels [410]. This conclusion has been confirmed by experiments which have shown that adult male rainbow trout exposed to 30 μg/L of octylphenol, nonylphenol or nonylphenoxy acetic acid, similar to levels found in rivers in the UK, Switzerland and the USA, produce the female egg yolk protein vitellogenin [411]. Another study which examined the effects of these same exposures on male rainbow trout has shown a reduction in testicular growth, and that octylphenol can increase vitellogenin production when present in the water at levels as low as 4.8 μg/L [412]. Similar effects have been seen in other fish species [403, 413]. Exposure of fish to higher levels of nonylphenol has been shown to result in testicular agenesis and impaired oogenesis [414].

Vitellogenin production in male rainbow trout has also been shown to be induced in the wild when fish are placed in rivers into which treated sewage flows [415, 416]. However in such mixtures other compounds contribute to the total estrogenic effect including low levels of the natural female hormones estradiol and estrone, and a small contribution from ethanyl estradiol [417]. A survey of wild fish (primarily roach) in UK rivers has found that a high percentage of males had eggs in their testes, in addition to female egg yolk protein in their blood [418]. These findings have been substantiated by a study in Japan that found similar physiological changes in wild fish species [419].

Nonylphenol affects testosterone metabolism and CYP450 expression in male rats at doses as low as 25 μg/kg [420]. Increased 5α-reductase activity and decreased formation of 16alpha-OH-, and alpha-OH-testosterone metabolites, and CYP2C and CYP3A protein were also observed. In rats exposed neonatally to 500 mg/kg/day NP abnormalities were observed in post pubertal reproductive function in female rats, and disrupted development of gonads occurred in both male and female rats [421].

I tested four different alkylphenol preparations in the cell culture assay. These were p-nonylphenol, p-octylphenol, and two ethoxylates, N-10 and N-100. In addition, nonylphenol was also tested in a two-generation transgenic mouse study to further investigate its effect upon androgen-regulated pathways.

4.2 Results

4.2.1 Nonylphenol

In vitro

p-Nonylphenol (NP) was tested for androgenic or antiandrogenic activity in PC-3 cells as described in the Materials and Methods. No consistent androgenic activity, in the form of increased firefly luciferase activity, was detected either in combination with DHT or when NP alone was added to the cells. However, a clear antagonist trend was seen and statistical analysis found that there was significant suppression of DHT induced, AR mediated firefly luciferase expression at NP concentrations above three micromolar in transfected PC-3 cells exposed to DHT (Figure 4-2A). However, when NP was tested for effects upon AR mediated gene transcriptional activity in LNCaP cells (experiments performed by Chris Butler) no effects were found either as an agonist with or without DHT, or as an antagonist of DHT (Figure 4-3).

When NP was tested for interaction with GR in PC-3 cells, it was found to produce no change in firefly luciferase activity (Figure 4-2B) either with or without dexamethasone. In contrast, when NP was tested for estrogenic or antiestrogenic activity in HepG2 cells as described, a significant increase in firefly luciferase activity resulted from the addition of NP but only at the 10μM concentration in the presence 17β-estradiol (Figure 4-2C). No effect was seen in the absence of 17β-estradiol.

When the ability of NP to compete with radiolabeled DHT for binding sites within PC-3 cells was studied, it was found that increasing the concentration of NP had no effect upon the amount of radiolabel bound within the cells (Figure 4-8B). A similar competitive binding assay was performed in PC-3 cells transfected with GR versus radiolabeled dexamethasone and again no competition was detected for cellular binding sites (Figure 4-8B).

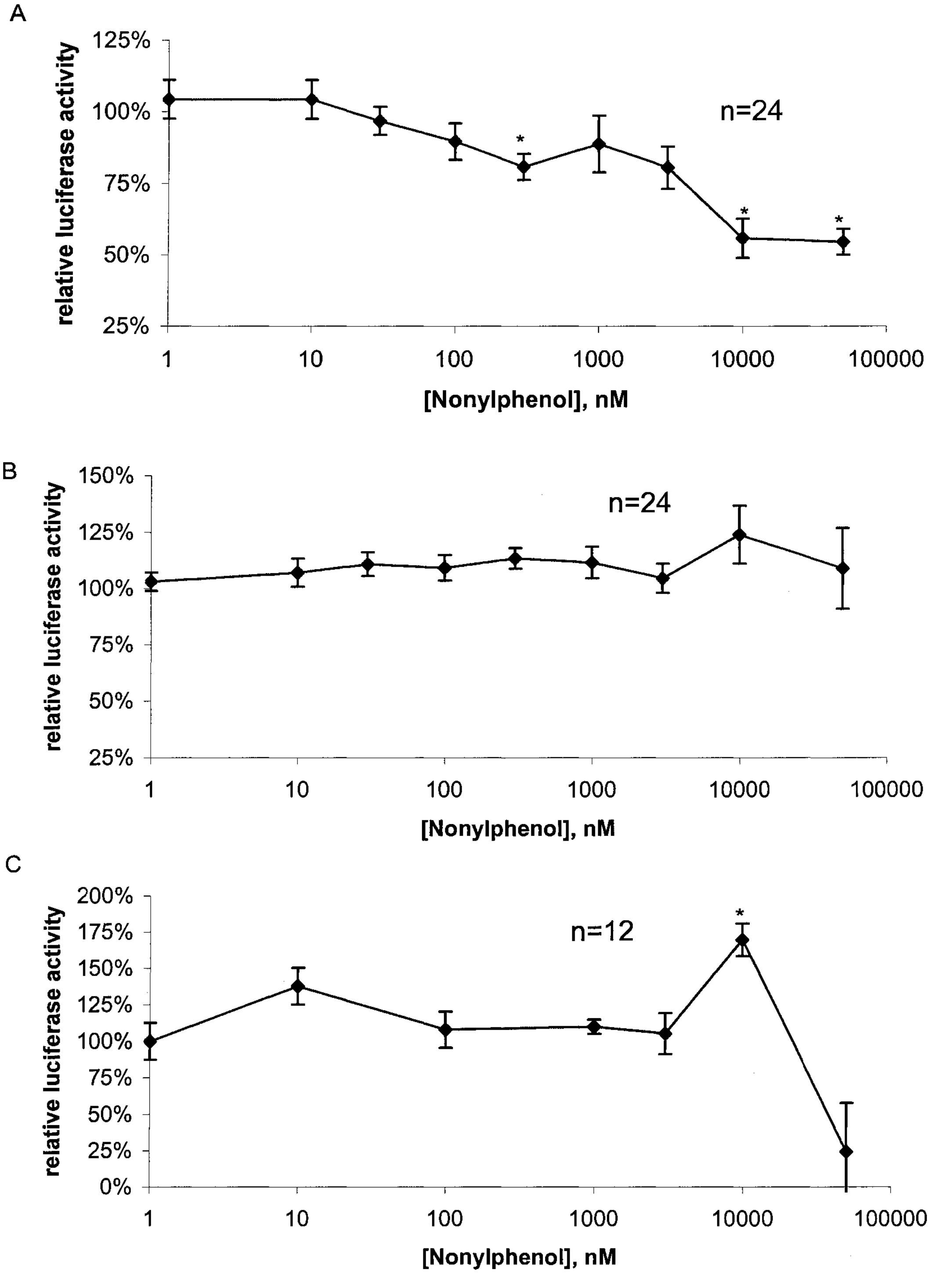


Figure 4-2: The effect of nonylphenol on steroid hormone mediated luiferase transactivation. The effects on AR with 1nM DHT in PC-3 cells (A); on GR with 1nM DEX in PC-3 cells (B); on ER with 1nM E2 in HepG2 cells (C) . The * indicates a value is significantly different ($p<0.05$, using Student's T-test) from control (no test compound added, set at 100% relative luciferase activity) . Number of individual wells (n=), from multiple experiments, used to derive the graph is shown. Error bars indicate the standard error of the mean (SEM).

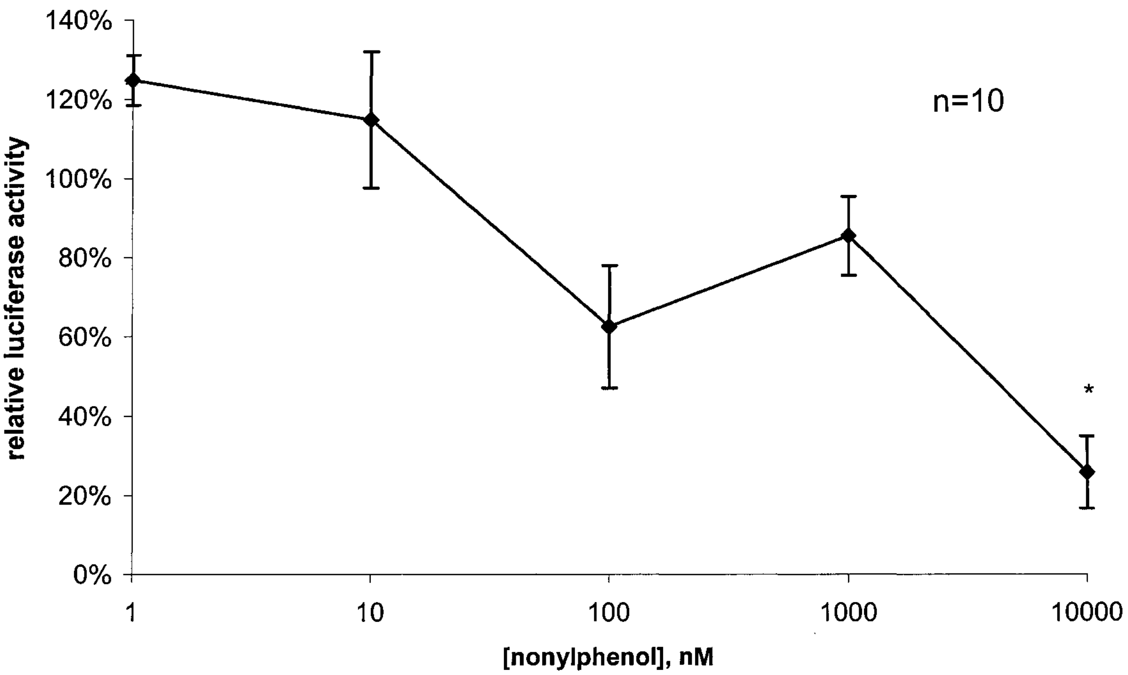


Figure 4-3: The effect of nonylphenol on androgen mediated luciferase transactivation in LNCaP cells. The * indicates a value that is significantly different ($p<0.05$, using Student's T-test) from control (no test compound added, set at 100% relative luciferase activity) . Number of individual wells (n=), from multiple experiments, used to derive the graph is shown.
Error bars indicate the standard error of the mean (SEM).

In vivo

Transgenic LPB-CAT mice were dosed with nonylphenol at 60 mg/kg/day by oral gavage as described in the Materials and Methods. A two-generation developmental study and an adult study were performed by Dr. Chris Butler. In addition, I repeated the adult male study and a parallel study with intact adult female mice. For the adult studies, male mice of a similar age were divided into two groups of 10 with half of the individuals in each group being castrated. One group was dosed with oil only, another with NP.

Body weight, organ weight for liver, heart, kidney, testes, epididymis and prostate as well as anogenital distance (AGD) were noted and serum was collected by cardiac puncture for analysis of hormone levels. In the developmental study, testis weight in NP-treated mice at eight weeks was significantly increased compared to controls and AGD increased but not significantly ($p=0.054$; Table 4-1). This correlated with an increased level of circulating thyroid hormone (Figure 4-4A) and increased CAT activity in treated animals (Figure 4-5). Thyroxine (T4) levels were also increased in intact adults treated with NP but not in castrates (Figure 4-4B). There was a tendency for treated animals to have elevated testosterone levels relative to the untreated animals at eight weeks (post-pubertal) but not at four weeks and this pattern was repeated in both of the adult studies although in no case was the increase statistically significant (Figure 4-6). In the second adult study, treatment with nonylphenol prevented a significant decline in body weight in the castrate mice during dosing (Table 4-2B)

		4 week old mice		**8 week old mice**	
		Control	**Treated**	**Control**	**Treated**
Testes		27.38±0.80	28.84±0.95	**41.02±1.60**	**45.93±1.16**
Prostate		2.23±0.19	2.11±0.27	4.68±0.27	4.47±0.34
AGD		56.0±1.4	55.6±2.4	50.05±0.24	54.09±0.32
Liver		**544.2±9.7**	**505.9±12.6**	493.8±10.8	497.4±13.5
Body weight	per pup	1.75±0.18	1.80±0.11	1.70±0.11	1.70±0.03
	per litter	**16.6±0.12**	**14.4±0.90**	14.5±1.23	18.8±0.35
	weaning	**6.93±0.30**	**8.61±0.57**	10.35±0.95	10.54±0.62
	autopsy	18.6±0.73	21.5±1.05	28.1±0.87	27.0±0.36

Table 4-1: Physiological parameters in mice exposed to nonylphenol.
Relative organ weights were derived by adjusting the individual organ weight for body weight. Developmental mouse study. Pairs of values in bold are statistically different ($p<0.05$) as determined by Student's T-test.

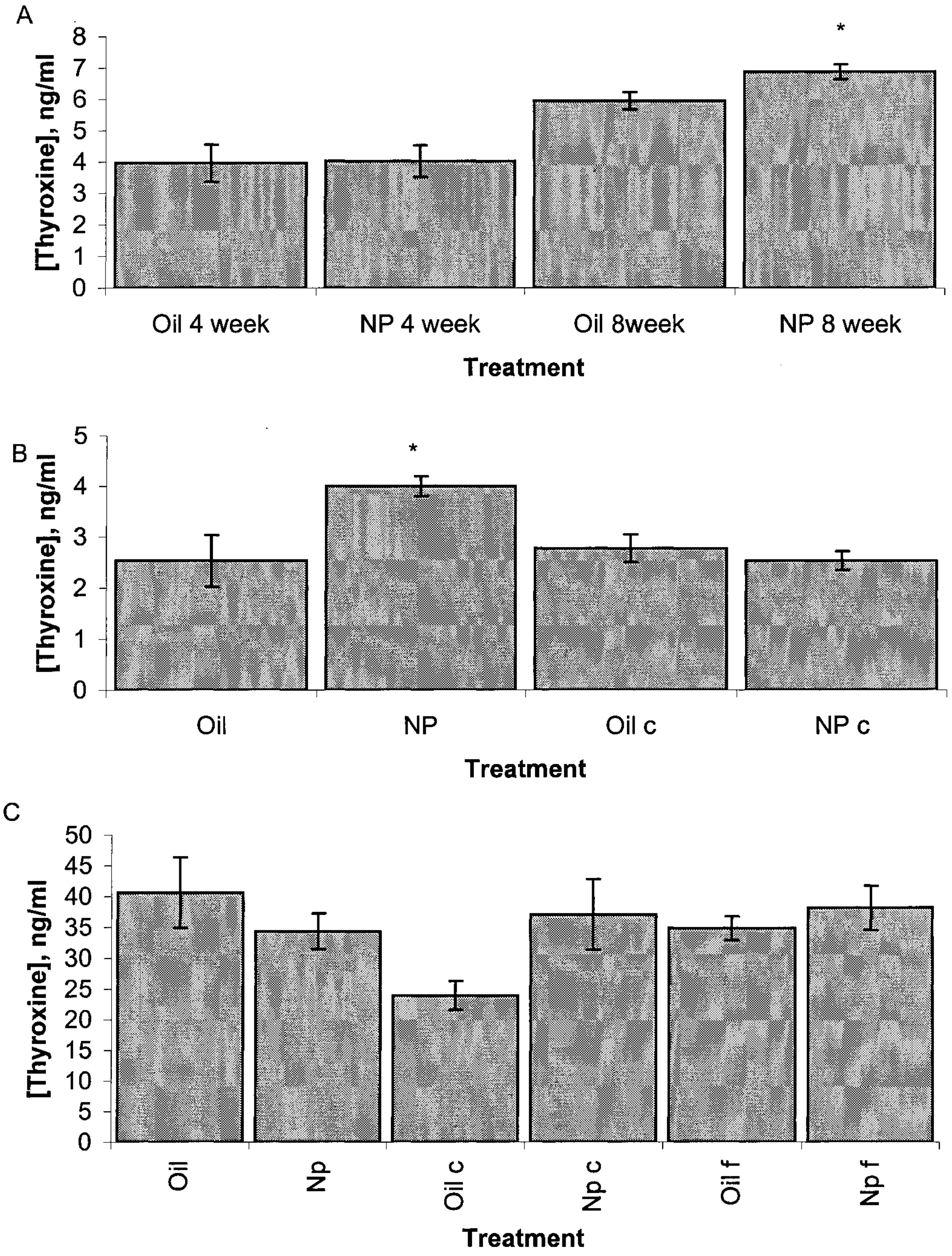


Figure 4-4: The effects of nonylphenol exposure on serum thyroxine levels in mice. Three separate studies were conducted; (A) shows the results from the developmental study, (B) shows the results from the adult study conducted by Dr. C. Butler, and (C) shows the results from my study. F denotes female, c denotes castrate male. NB a log scale was used for the hormone concentration. The * indicates a value is significantly different ($p<0.05$, using Student's T-test) between the dosed and control groups. Error bars indicate the standard error of the mean (SEM).

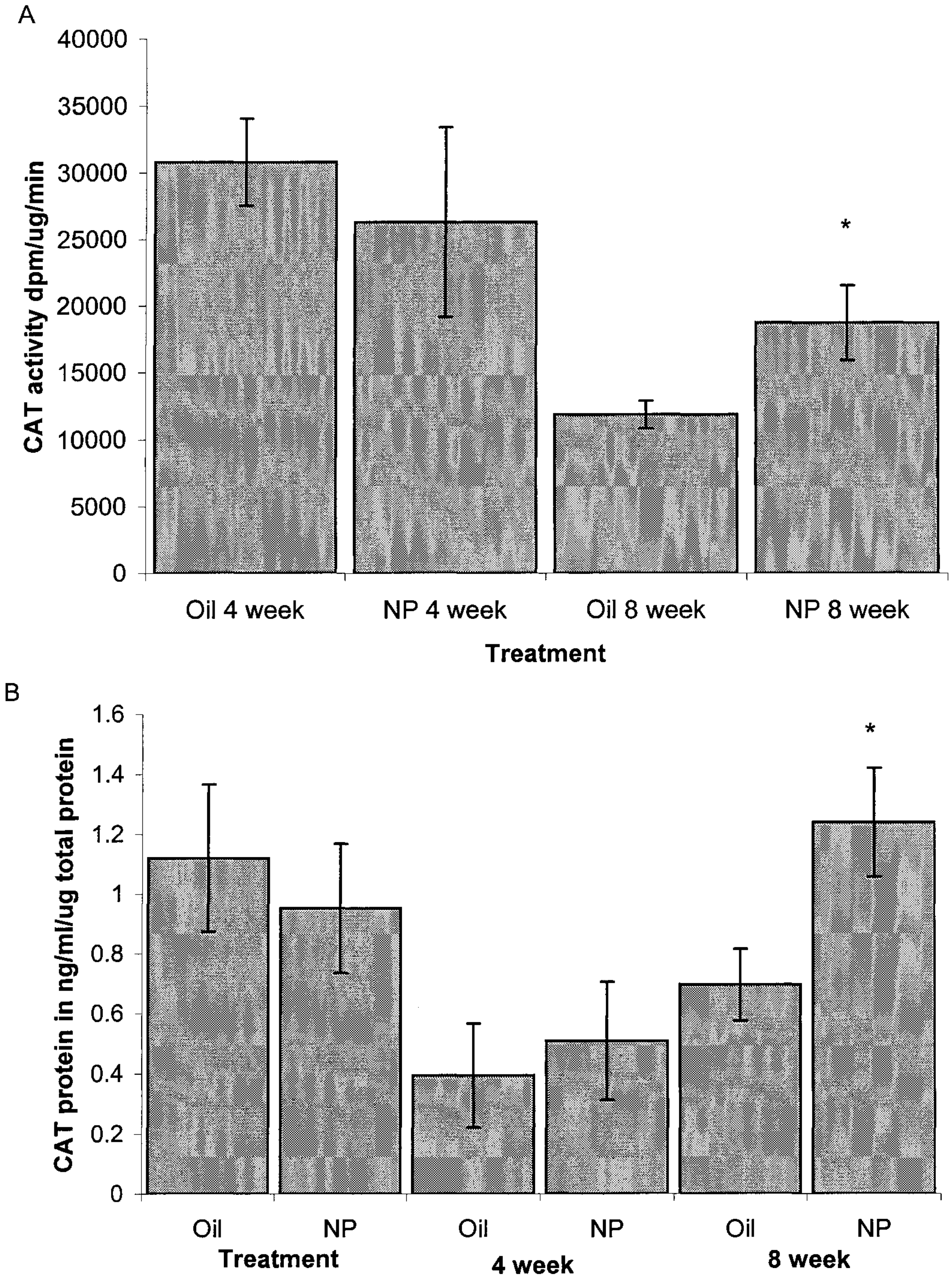


Figure 4-5: CAT activity and protein levels in mouse prostates after treatment with nonylphenol or oil (Performed by Dr. C. Butler). Enzyme activity assay (A) and ELiSA for CAT protein (B). The * indicates that the treatment group is statistically different from its control (p,0.05, using Student's T-test). Error bars indicate the standard error of the mean.

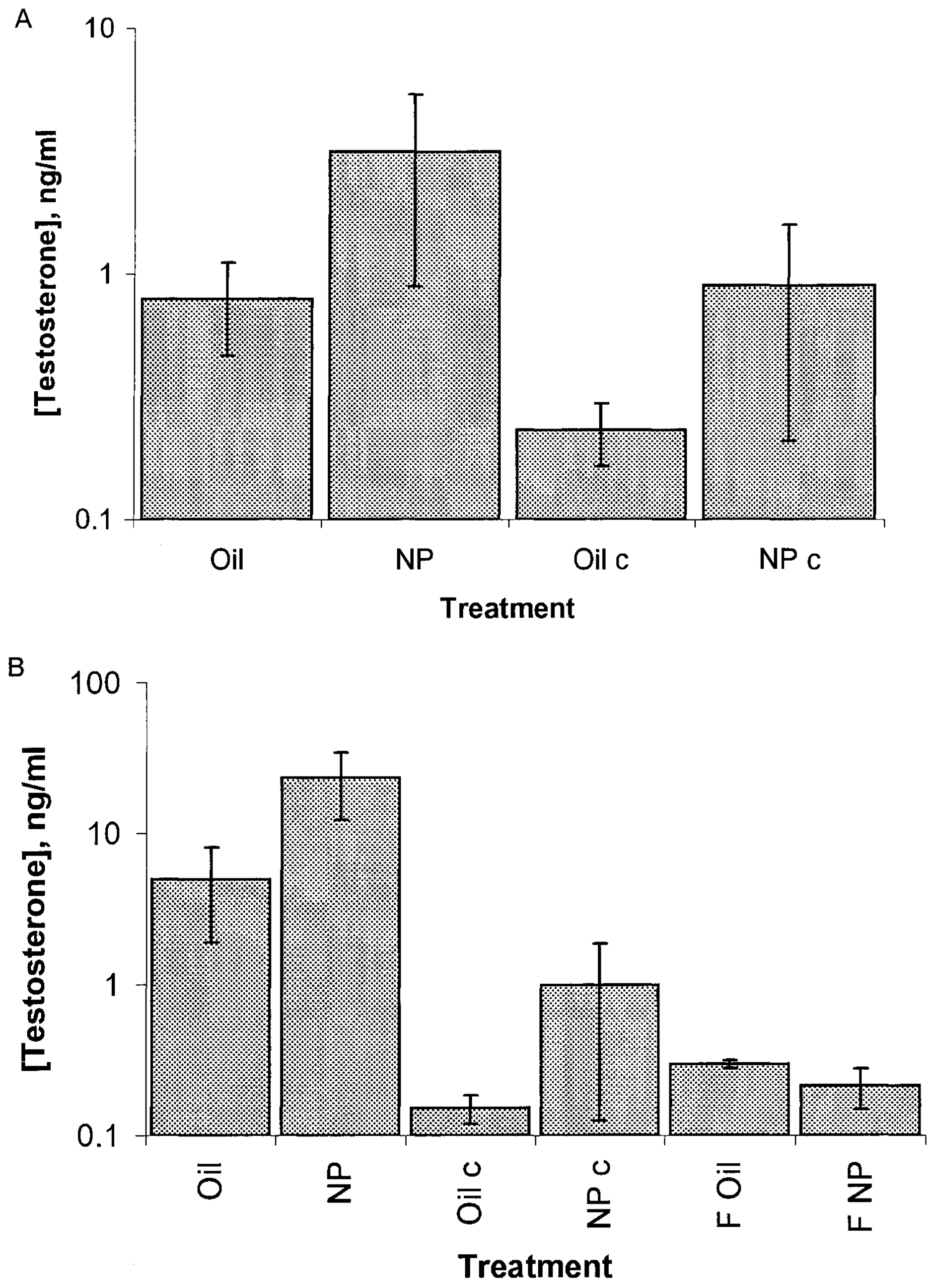


Figure 4-6: The effects of nonylphenol exposure on serum testosterone levels in adult mice. Two separate studies were undertaken. The results in A were from a study conducted by Dr. C Butler and those in B from my study. F denotes female, c denotes castrate male. Error bars indicate the standard error of the mean. NB a log scale was used for the hormone concentration.

A		**Intact**		**Castrate**	
		Control	**Treated**	**Control**	**Treated**
Liver		401.08±26.39	372.21±14.92	416.43±18.10	416.78±13.22
Prostate		5.56±0.84	5.16±0.44	1.19±0.26	1.21±0.24
Body weight	at castration	38.12±1.63	39.76±1.87	37.36±1.03	40.18±2.09
	at autopsy	38.20±3.25	39.40±1.88	33.18±1.34	35.54±1.48
	change	0.08	(0.36)	(4.18)	(4.64)

B		**Intact**		**Castrate**	
		Control	**Treated**	**Control**	**Treated**
Liver		1628.7±60.5	1825.1±91.6	**1710.6±76.1**	**1989.6±34.5**
Prostate		18.9±1.36	17.2±1.82	**8.4±0.94**	**4.8±0.71**
Body weight	at day 1	34.5±2.7	32.5±2.3	41.8±5.1	37.5±3.9
	at autopsy	37.5±2.56	36.1±2.27	38.6±3.22	41.4±4.58
	change	3.0±5.26	3.6±4.57	(3.2) ±8.32	3.9±8.48

Table 4-2: Physiological parameters in mice exposed to nonylphenol.
Adult mouse studies. Relative organ weights were derived by adjusting the individual organ weight for body weight. Data in Table A is from the experiment conducted by Dr. C. Butler, while Data in Table B contains data from my adult study. Paired values in bold are significantly different from each other ($P<0.05$) as determined by Student's T-test.

4.2.2 Octylphenol

When p-octylphenol (OP) was tested for interaction with AR, and GR in PC-3 cells as described in the Materials and Methods, no consistent effect, in the form of changes in firefly luciferase activity, was detected either in combination with DHT or DEX (Figure 4-7). Testing for estrogenic or antiestrogenic activity in HepG2 cells showed an increase in firefly luciferase activity resulted from the addition of 10 μM OP in the presence 17β-estradiol (Figure 4-7C). No effect was seen in the absence of 17β-estradiol (results not shown).

When the ability of OP to compete with radiolabeled DHT for binding sites within PC-3 cells was studied it was found that increasing the concentration of OP apparently reduced the amount of radiolabel bound within the cells although this decrease was not statistically significant (Figure 4-8C). A similar competitive binding assay was performed with NP in PC-3 cells transfected with GR versus radiolabeled dexamethasone and again failed to show statistically significant competition for cellular binding sites (Figure 4-8C).

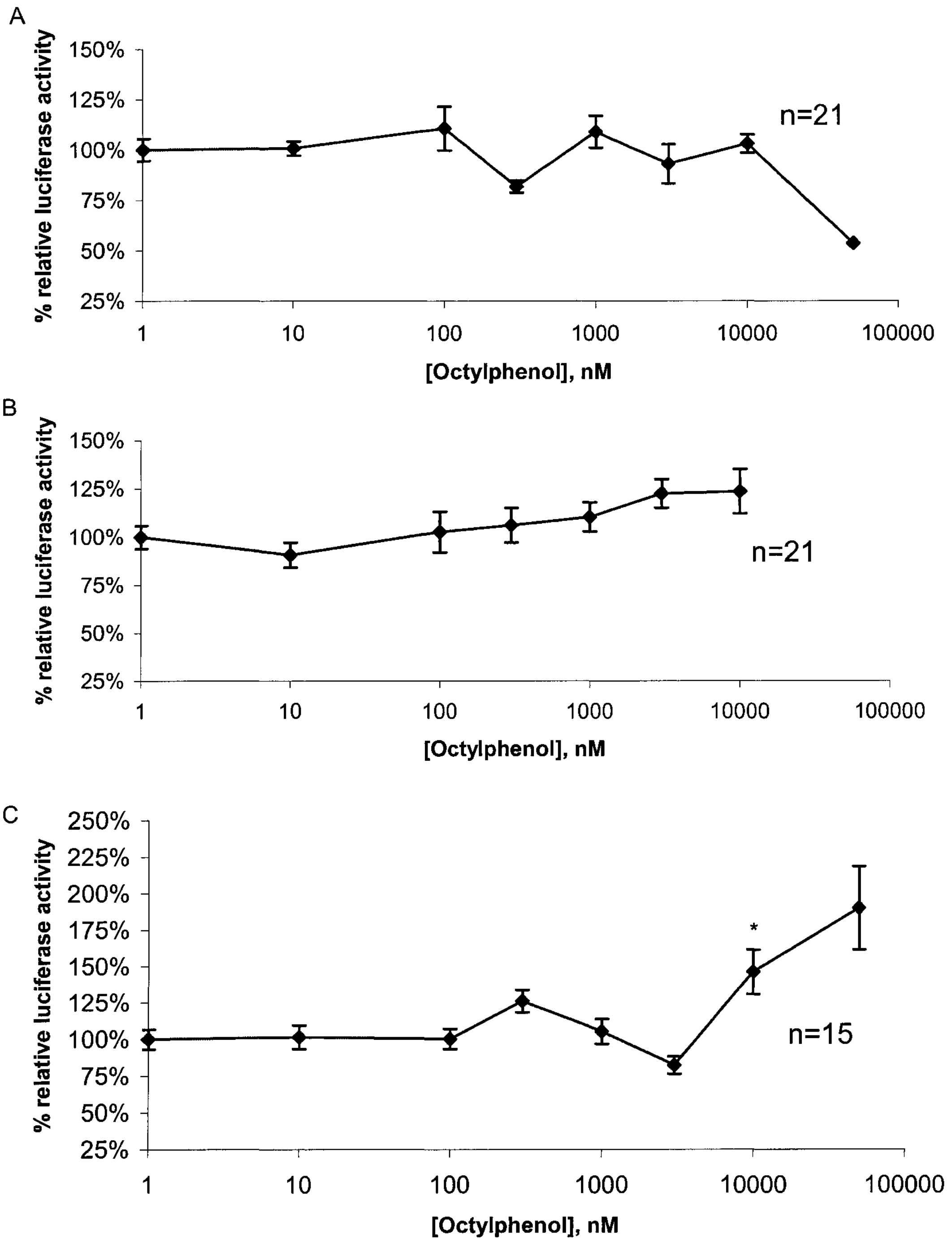


Figure 4-7 : The effects of octylphenol on receptor mediated luciferase transactivation. The effects on AR with 1nM DHT in PC-3 cells (A); on GR with 1nM DEX in PC-3 cells (B); on ER with 1nM E2 in HepG2 cells (C). The * indicates a value is significantly different ($p<0.05$, using Student's T-test) from control (no test compound added, set at 100% relative luciferase activity) . Number of individual wells (n=), from multiple experiments, used to derive the graph is shown. Error bars indicate the standard error of the mean (SEM).

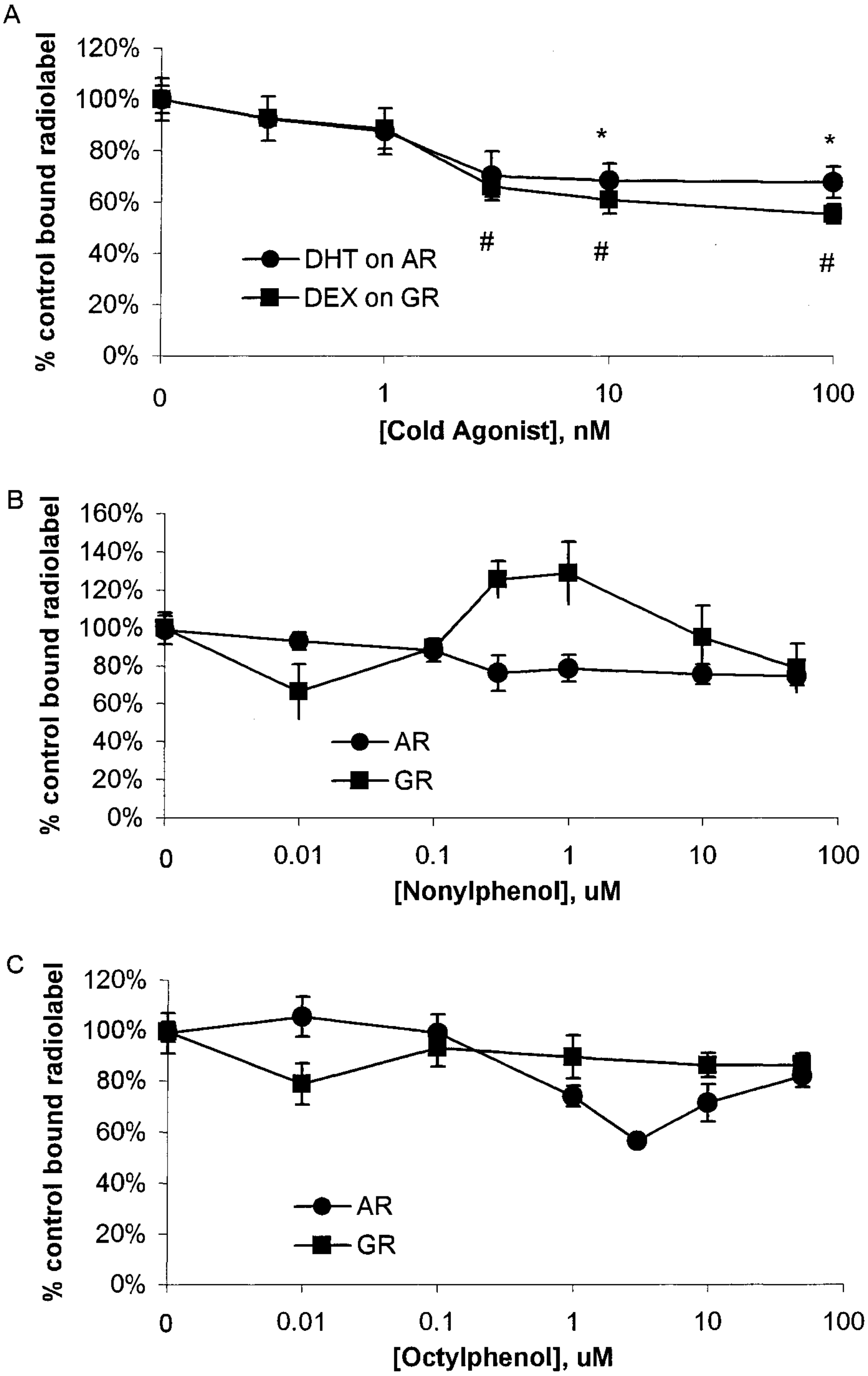


Figure 4-8: The ability of alkylphenols to compete with known agonists for steroid receptor binding sites. (A) Cold versus radiolabelled DHT and DEX in AR and GR transfected PC-3 cells respectively. (B) Nonylphenol versus radiolabelled DHT and DEX in AR and GR transfected PC-3 cells respectively. (C) Octylphenol versus radiolabelled DHT and DEX in AR and GR transfected PC-3 cells respectively. Error bars indicate the standard error of the mean (SEM). Statistically different values for AR (*) and GR (#)are indicated (p,0.05, using Student's T-test).

4.2.3 Nonylphenol Polyethoxylates

When nonylphenol polyethoxylate (NPE) N-10 was tested in the AR PC-3 cell culture reporter assay a significant antagonist response versus 1 nM DHT was seen at concentrations above 3 μM (Figure 4-9A). When a similar assay was performed using GR transfected PC-3 cells with and without 1 nM DEX no effect was seen in response to N-10 (Figure 4-9A).

Testing of a longer chain NPE, N-100, in the AR and GR reporter gene assays in PC-3 cells indicated agonist activity with both receptors (Figure 4-9B). With AR, luciferase activity was significantly elevated when concentrations above 1 μM were added, whereas for GR a significant agonist response was only seen at 100 μM.

4.3 Discussion

The above work demonstrates that male mice exposed to p-nonylphenol throughout their development exhibit some physiological changes compared with controls including elevated serum T4 and testosterone levels, increased testis size, CAT activity, and anogenital distance. Exposure in adult mice was also found to impact some physiological parameters. Effects upon steroid receptors were seen with the various alkylphenol products tested in the *in vitro* assays used. It is likely that many of these *in vitro* and *in vivo* outcomes are linked. The testes are the primary sites of testosterone production in the adult mouse, but not a major target for it; hence an increase in testis size may lead to an increase in testosterone production. The probasin promoter regulating the CAT reporter in LPB-CAT mice is prostate specific and is induced exclusively by androgens, therefore the increase in CAT activity is likely the direct result of an increase in serum testosterone, supporting the observation of an increasing trend even though it was not statistically significant. Similarly anogenital distance is also an androgen dependent characteristic and the slight increase seen in this parameter is likely also a direct result of elevated testosterone and may indicate that testosterone levels are in fact elevated at a relatively early stage since it appears anogenital distance is determined during mid-gestation [422-424].

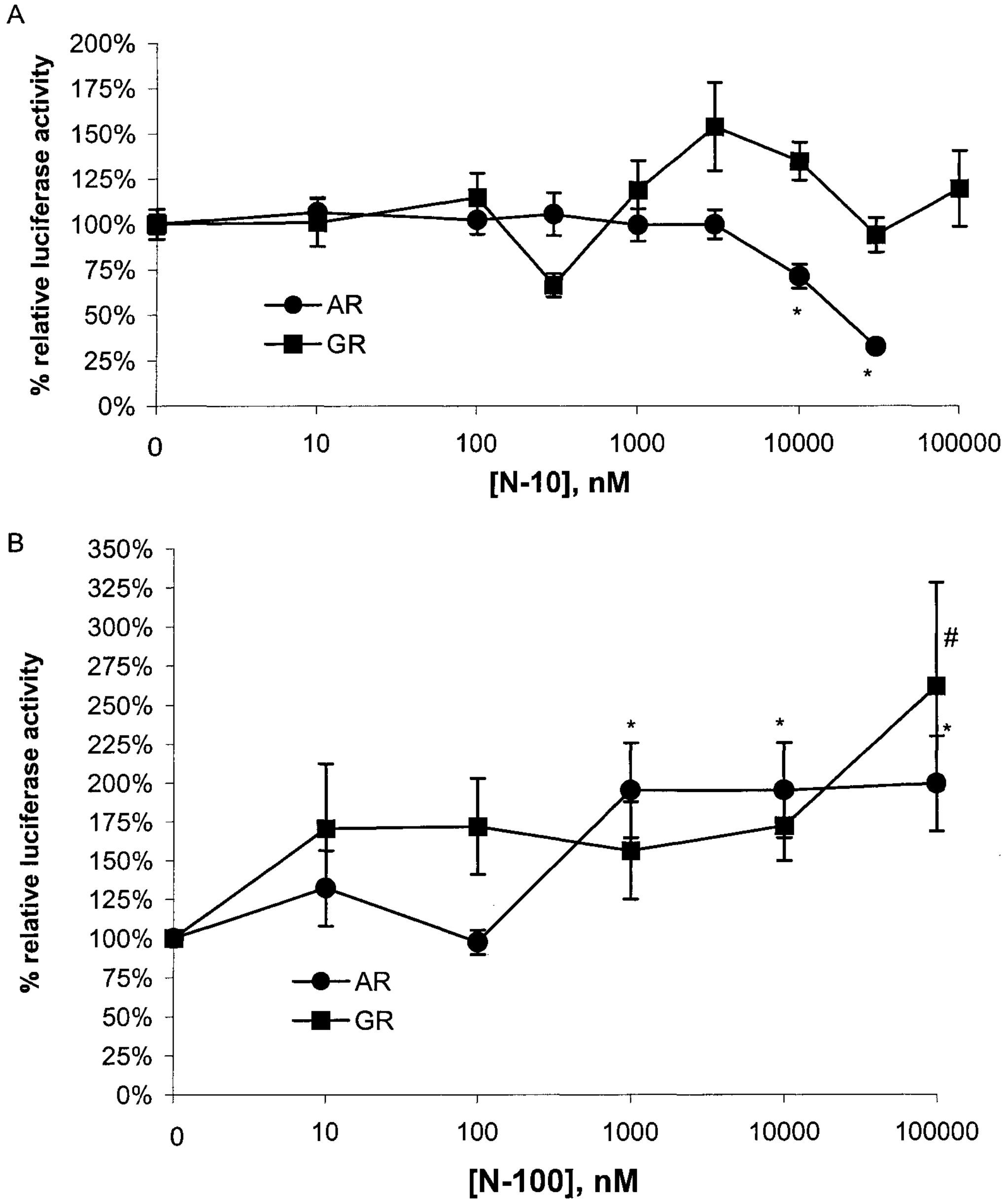


Figure 4-9: The effects of alkylphenol polyethoxylates on receptor mediated luciferase transactivation. N-10 (A), and N-100 (B) were added to transfected cells in culture and luciferase product was measured after incubation in combination with 1nM known agonist. Error bars indicate the standard error of the mean (SEM). Values that differ significantly from the control are indicated by * for AR and # for GR ($p<0.05$, using Student's T-test).

The *in vitro* work indicates that NP may have antiandrogenic effects, while both it and possibly octylphenol are ER-α agonists. In addition, both of the APEs tested act as AR agonists. If NP acts as an AR antagonist *in vivo* then it may interfere with androgen mediated negative feedback regulation of GnRH release from the hypothalamus and perhaps gonadotropin release from the pituitary. As a result, there would be an inability to respond to rising serum testosterone by adequately down-regulating the release of these hormones and that could result in increased secretion of LH/FSH from the pituitary and ultimately elevated testicular hormone synthesis and release.

Xenobiotic compounds, such as polychlorinated biphenyls, that affect thyroid gland/thyroid hormone function have been shown to cause hyperplasia of the testis [425] NP has also been shown to cause Sertoli cell hypertrophy in fish [426]. In these experiments, NP has increased testis size in treated mice possibly through a mechanism involving thyroid hormone or its receptor. To our knowledge, this is the first report of an effect on thyroid hormone status by nonylphenol.

Nonylphenol exposure affects testis size in animals treated to eight weeks of age. Testis weight in NP-treated mice at eight weeks was significantly larger than in controls. This correlated to an increased level of circulating thyroid hormone (T4) and increased CAT activity in treated animals at eight weeks. T4 levels were also increased in intact adults treated with NP. Though not statistically significant, there was a tendency for treated animals to have higher average testosterone levels than untreated animals at eight weeks (post-pubertal) but not at four weeks. Elevated testosterone may also account for the apparent protective effect of NP treatment against castration induced weight loss in adult mice.

Thyroid hormone receptors have been found in Leydig, Sertoli and peritubular cells of the testis [427-430]. Induced hypothyroidism leads to decreased FSH and LH concentrations in serum and delayed maturation of the testis [431] and decreased testis size [432] but can also lead to increased testis size [425, 433, 434]. Triiodothyronine (T3) administered to rats causes decreases in estrogen receptor content of the Sertoli cells of the testis [432, 435], increases androgen receptor content in peripubertal Sertoli cells and reduces basal and FSH-induced aromatase activity in cultured Sertoli cells [436]. These changes are age dependent and T3 administration or changes in thyroid status cause minimal effects if they occur post-pubertally [430, 437]. T3 administration in neonatal rats leads to decreased testis size and early maturation of adult type Leydig cells in the testis with an accompanying decrease in testosterone production [438], but has also been shown to increase testis size, stimulate spermatogenesis and development of interstitial cells [439]. In humans, thyrotoxicosis (hyperthyroidism) in girls causes delayed sexual maturation but there have been no studies showing the effects of this condition on gonadal function in boys [430].

Effects on testis size appear to differ depending on the severity and duration of hyper or hypothyroidism induced. Mild hyperthyroidism accelerates growth whereas severe hyperthyroidism causes growth retardation [440]. Duration of exposure also appears to affect the outcome of thryroid hormone changes. Chronic hypothyroidism leads to delayed maturation of seminiferous tubules and reduced testis size whereas transient hypothyroidism leads to increases in adult testis size. The critical period appears to be the first week of post-natal life. T3 administration, in contrast, transiently increases testis size and longer exposure accelerates testis development leading to early maturation of Sertoli cells and eventual decrease in adult testis size [441].

Chapter V - Polychlorinated Biphenyls

5.1.1 Industrial Use of Polychlorinated Biphenyls

Polychlorinated biphenyls (PCBs) were first manufactured in the USA in 1929 and quickly entered widespread commercial use, mainly as coolants and lubricants in electrical equipment. In addition, PCBs also found utility in a host of general use products including solvent extenders, flame-retardants, inks, dyes, paints, and adhesives, and were found in carbonless copy paper, newsprint, and caulking compounds. Annual usage of PCBs peaked in 1970 at approximately 40 million kilograms in the United States. Up to the prohibition of PCBs by the USA in 1977 it is estimated that more than ten times this amount had been manufactured [236].

PCBs were not produced as pure compounds, rather they were manufactured as mixtures of isomers or congeners by the substitution of chlorine atoms for the hydrogens on a biphenyl core, to make them suitable for each particular application. Each mixture contains a varying amount of the 209 different possible congeners. The congeners vary in the number and position of chlorine substitutions, affecting their physical and biological properties. In the US, the Monsanto Company was the largest producer of PCBs and marketed a range of mixtures under the trade name Aroclor. Each Aroclor was numerically identified according to the number of carbons in the core structure and the percent chlorine content by weight. Therefore, Aroclor 1254 contained congeners with a 12-carbon backbone biphenyl ring (Figure 5-1), and was composed of 54% chlorine by weight.

5.1.2 Metabolism and Half Life

Decomposition of PCBs in the environment is a complex issue. Some reports suggest that complete degradation of all PCB congeners is possible [442, 443] but typically this requires specific conditions, such as those created in a lab and not found in most real world settings. Environmental monitoring data indicate that although overall PCB contamination levels are declining, many individual congeners are persisting in the environment suggesting that breakdown by natural processes is limited for some congeners [236]. Under suitable conditions PCBs can undergo hydroxylation or photo-degradation in the atmosphere or in water [444-447] but the rate by which these processes

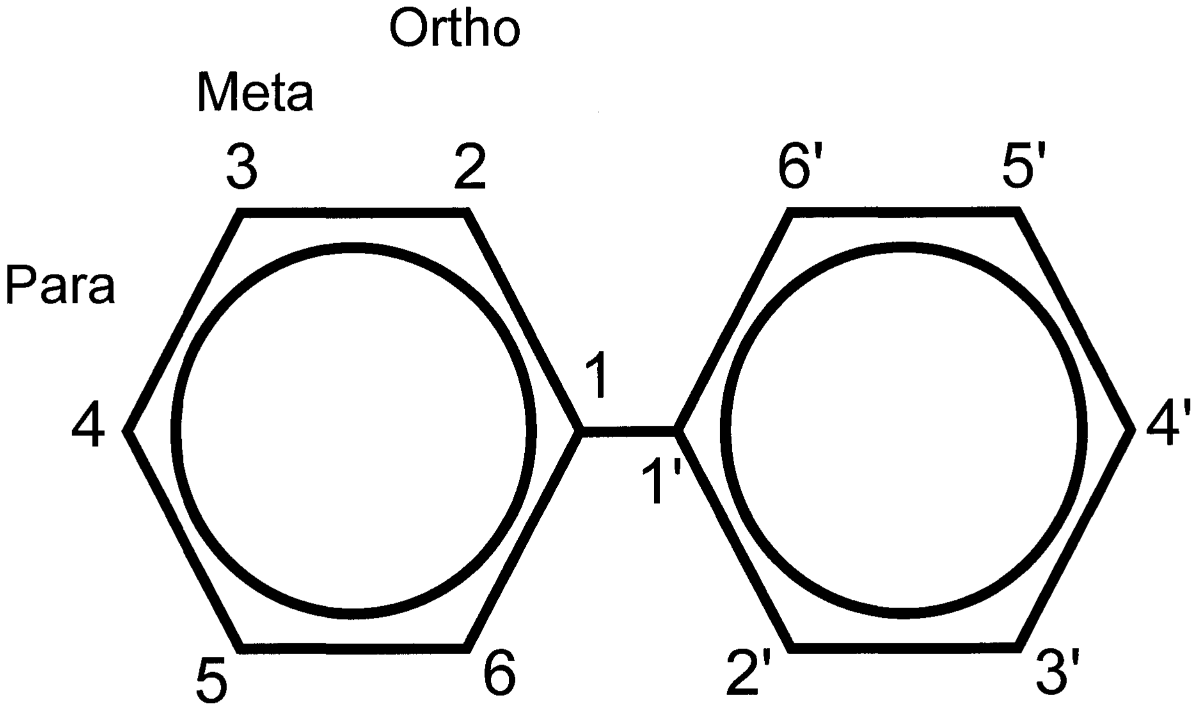


Figure 5-1: PCB structure

eliminate PCBs varies greatly according to local conditions. Microbial metabolic pathways can partially degrade PCBs via anaerobic dechlorination especially at the meta- and para- positions [443, 448]. Aerobic microbial degradation may act on ortho-substituted congeners and ultimately lead to degradation of the biphenyl core in dechlorinated or less chlorinated congeners [442, 443]. Due to their hydrophobic nature, PCBs readily bind to particulate matter within bodies of water. As a result, another significant method of PCB elimination from the environment is loss to sediment. This may be particularly important in areas such as the Great Lakes where the deep sediments are relatively undisturbed and the low water temperature does not favour bacterial and environmental breakdown.

Like other hydrophobic contaminants, environmental PCB levels are biomagnified as they concentrate in animals with each successive step up the food chain. Slow accumulation of PCBs in adipose tissues over time produces tissue concentrations far above any levels detected in the blood immediately after acute exposure. The organisms in each trophic level absorb and store the majority of their accumulated exposure from food [449, 450]. For example, in the Lake Michigan food web, PCB concentrations increase 12.9 times from plankton to fish [451].

As with metabolism, the accumulation of congeners is influenced by their individual structure. Generally as the degree of chlorination of a congener increases so does uptake through the intestine while availability to metabolism and elimination decreases. Experiments with radiolabeled PCBs given to various laboratory animals indicate that uptake through the gastro-intestinal (GI) tract is very efficient. For mono-, di, and tri-chlorobiphenyls values in the range of 70-90% are typically reported. For the more highly chlorinated congeners 95 to 98% of the dose is absorbed [452, 453]. From here, the ingested PCBs associate with fats absorbed from the diet and protein and lipid components of the serum to enter the circulatory system. The first site reached by this PCB-enriched blood is the liver, which is able to begin processing some of the congeners. The metabolite products along with those PCBs that cannot be readily processed by the liver are redistributed by the circulatory system. PCBs become incorporated into lipoproteins and may bind to other serum transport proteins (e.g. steroid hormone binding globulin, or transthyretin) that convey them through the blood and assist in their deposition into various tissues. Due to their lipophilic nature most ultimately concentrate in lipid rich tissues, especially adipose and skin [454] where they accumulate to concentrations greatly in excess of serum concentrations [236].

Over time and without new or continuing exposure, congeners that can be metabolised and excreted become depleted from an organism while those congeners that are not metabolised persist and accumulate with subsequent exposures. As a result the pattern of congeners detected in an organism's adipose tissue diverges from the profile of the PCB mixture to which that organism has been exposed. Consequently as a PCB mixture proceeds through the food web to subsequent trophic levels it becomes increasingly depleted for certain congeners while others persist (Table 5-1). Hence the concentration of any individual PCB congener in the tissue of an exposed animal will be a function of the level of exposure, the ability of the organism to clear the PCB, and the time elapsed since the exposure. This is well demonstrated in a study of the tissues of rats dosed with Aroclor 1254. Relative to Aroclor 1254, the brain, liver, blood, and adipose tissue contained a lower concentration of tetra- and penta- congeners, and a greater concentration of hexa- to nona- congeners [455].

PCB Reservoir	PCB Exposure	Absorption Pattern	Metabolic pattern	Adipose Store
Sediment	A-L	All Bind	No degradation	
Nematode	A-L	A-K	A & E	B-D, F-K
Fish	B-D, F-K	B-D, F-K	B, D & F	C, G-K
Mammal	C, G-K	C, G-K	G & H	C, I, J, K

Table 5-1: A model of congener depletion.
Congener depletion varies in an environmental mixture representing a selection of congeners with varying chlorine substituents as it moves up through different organisms in the food web. PCB containing sediment is ingested by a nematode, which in turn is eaten by a fish, which is eventually consumed by a mammal. At each stage, it is assumed that dosing is a temporal event and that sufficient time passes for the organism to fulfil its metabolic potential before being predated and passing on the PCBs to the next trophic level. Of 12 initial congeners, called A to L, contaminating the environment only 4 (C, I, J and K) are ultimately detected in the mammal's adipose tissue. One congener (L) was never efficiently absorbed since it was tightly bound to lake sediments, while A, B, D-H are all metabolised and eliminated by the various organisms through which they pass. Adapted from Safe [456].

5.1.3 PCB Metabolism

Polychlorinated biphenyls are very hydrophobic, while biological excretory mechanisms are based upon aqueous transfer of unwanted material. Clearly native PCB congeners will not be readily excreted. Even if they were somehow transferred to excretory regions of the liver and kidney and ultimately the bladder or GI tract, their lipophilicity would favour their rapid partition from aqueous excreta back into the surrounding cellular material and ultimately back into the circulatory system. Consequently, they must undergo chemical modification to facilitate their removal from the body.

The primary site of PCB and xenobiotic metabolism is the liver, although some can occur at other sites such as the kidney [457]. In eukaryotic cells metabolism primarily occurs inside microsomes or in the mitochondria. Within these structures the metabolic enzymes are closely associated with the membranes and so are brought into intimate contact with the lipophilic PCB substrates [450]. Xenobiotic metabolism is occurs in two phases. Phase I metabolism is found in all living organisms, and involves alteration of the basic chemical structure of a substrate, for example the oxidation of a double bond. Phase

II metabolism is a speciality of multicellular organisms and involves the conjugation of various substituents onto reactive groups in the target molecule.

Phase I Metabolism

There are no comprehensive studies of all 209 PCB congeners in any one organism or species; however, sufficient studies have been performed upon certain sets of congeners to enable the most significant metabolic pathways to be elucidated. Almost universally in animals, the initial steps in PCB metabolism are performed by the hepatic cytochrome P450 (CYP) enzymes. The biphenyl linkage directs metabolism to the ends of the molecule and the position of chlorine substituents on the phenyl rings determines the sites of oxidative attack [456]. CYP 1A1 catalyses oxidation at carbon atoms and is likely the most important enzyme in initiating metabolism of the co-planar PCBs, which in turn can induce CYP 1A1 expression. CYP 1A2 although induced by some congeners primarily oxidises at nitrogen or sulphur and therefore is unlikely to be important in PCB metabolism [458]. The CYP 1A oxidation yields an arene oxide intermediate that is reduced spontaneously or by epoxide hydrolase to 2- and 4-hydroxybiphenyls. The formation of 3-hydroxybiphenyl product is thought to be produced by a direct oxygen insertion pathway that remains to be elucidated fully, but may involve CYP 2B and proceeds at a much slower rate than CYP 1A mediated metabolism [458]. The arene oxide products of the CYPs are reactive intermediates thought to partially account for the hepatic toxicity and carcinogenicity that has been associated with PCBs in animal experiments. There is evidence to suggest that dihydroxy metabolites of PCBs with low chlorine content are activated to reactive intermediates that produce oxidative DNA damage [459]. These results provide a possible mechanism in support of the hypothesis that exposure to PCBs contributes to the incidence of breast cancer in humans.

Chlorine substitution on a carbon atom effectively blocks metabolism at that position. The presence of the relatively large chlorine atom on the outside of the ring results in steric hindrance of the enzyme active site access to the phenol ring at that position and also reduces the localised electron density reducing the potential for nucleophile attack. Consequently the most readily metabolised PCBs are those with vicinal-unsubstituted carbons especially at the 3 and 4, or 4 and 5 positions [460].

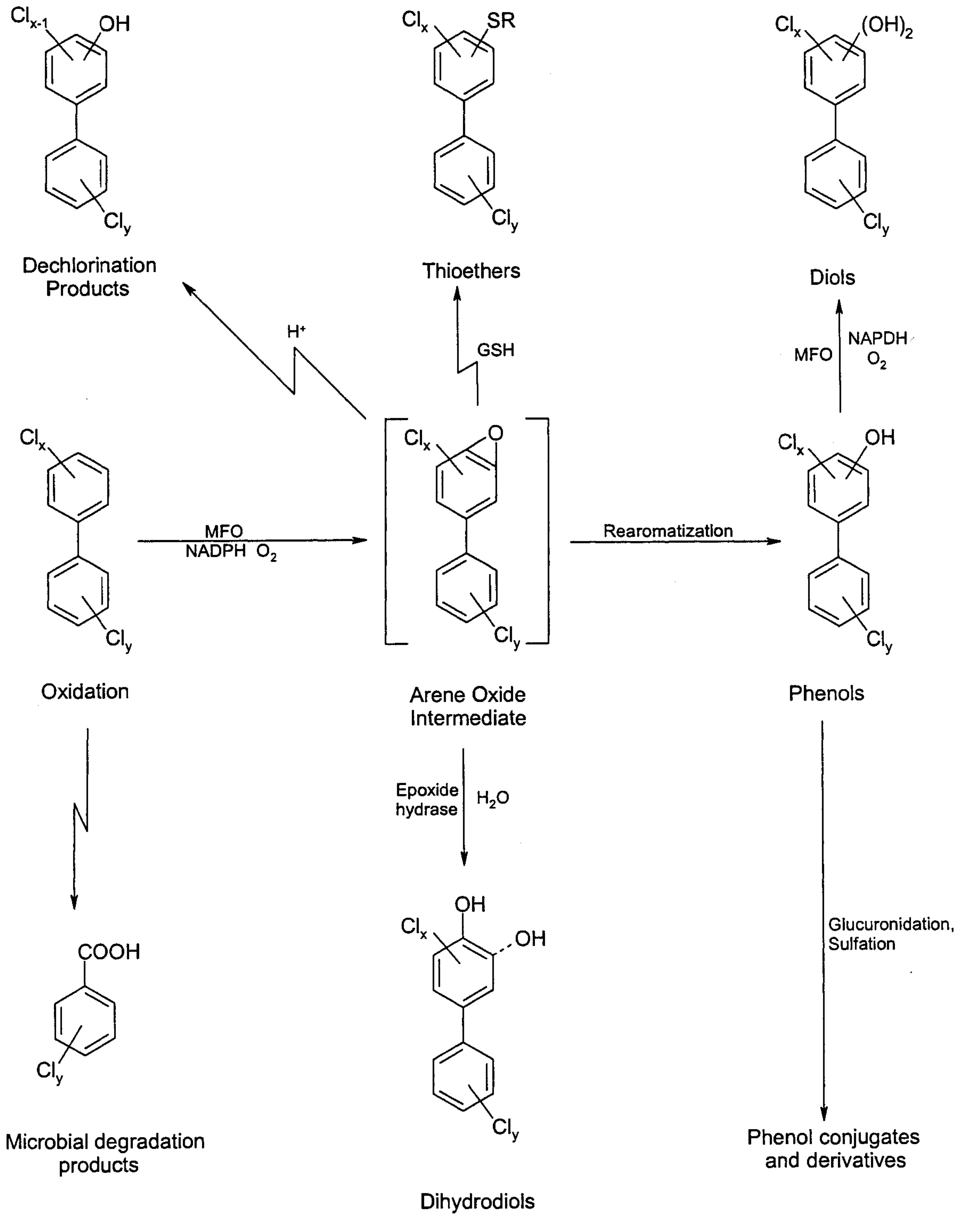


Figure 5-2: Metabolic pathways for polychlorinated biphenyls in mammals.
MFO (mixed function oxidase) = P450 enzyme oxidation, GSH = Glutathione. From Safe [456].

Where the four position is occupied, vicinal-unsubstituted carbons at other locations are also favoured targets for CYP mediated oxidation [456].

Safe elucidated a generalised set of guidelines for PCB congener metabolism based upon the animal data available and generated a summary of the major metabolic pathways ([456]; Figure 5-2). Hydroxylation is favoured in the para position in the least chlorinated phenol ring unless this site is sterically hindered (i.e. 3,5-dichlorobiphenyl). In the lower chlorinated biphenyls, the para position of both phenyl rings and carbon atoms that are para to the chloro substituents are all readily hydroxylated. The availability of two vicinal unsubstituted carbons (particularly C4 with C5 or C3) also facilitates oxidative metabolism of the PCB substrate but is not a necessary requirement for metabolism. As the degree of chlorination increases on both phenyl rings, the rate of metabolism decreases. The metabolism of specific PCB isomers by different species can result in considerable variations in metabolite distribution

In the simplest example studied [461] 4-chlorobiphenyl metabolism is exclusively directed to the unsubstituted phenyl ring where hydroxylation at the para position produces a single 4-hydroxy, 4'-chlorobiphenyl product in all species studied. This pattern is repeated in any case where only one phenyl ring is chlorinated. The ease of metabolism of this class of PCBs likely explains their almost complete absence from environmental samples.

Phase II Metabolism

Phase II metabolism consists of a host of reactions performed by specialised enzymes resulting in the conjugation of different side chains to, or modification of, the reactive groups generated by phase I metabolism. Unlike the cytochromes, which are found in many prokaryotes, the enzymes in phase II metabolism are found exclusively in eukaryotes. Two of the most common phase II conjugation reactions are mercapturation and glycosylation.

Mercapturation

Epoxides make excellent substrates for the enzyme glutathione-S-transferase that introduces the tripeptide glutathione (glutamyl-cysteinyl-glycine) to the phenol ring. The conjugated product is frequently processed further via hydrolysis by glutamyl transferase, then acetylation to a mercapturic acid by N-acetyl transferase. Degradation can proceed further as shown in Figure 5-3 to finally yield a methyl sulphone [450].

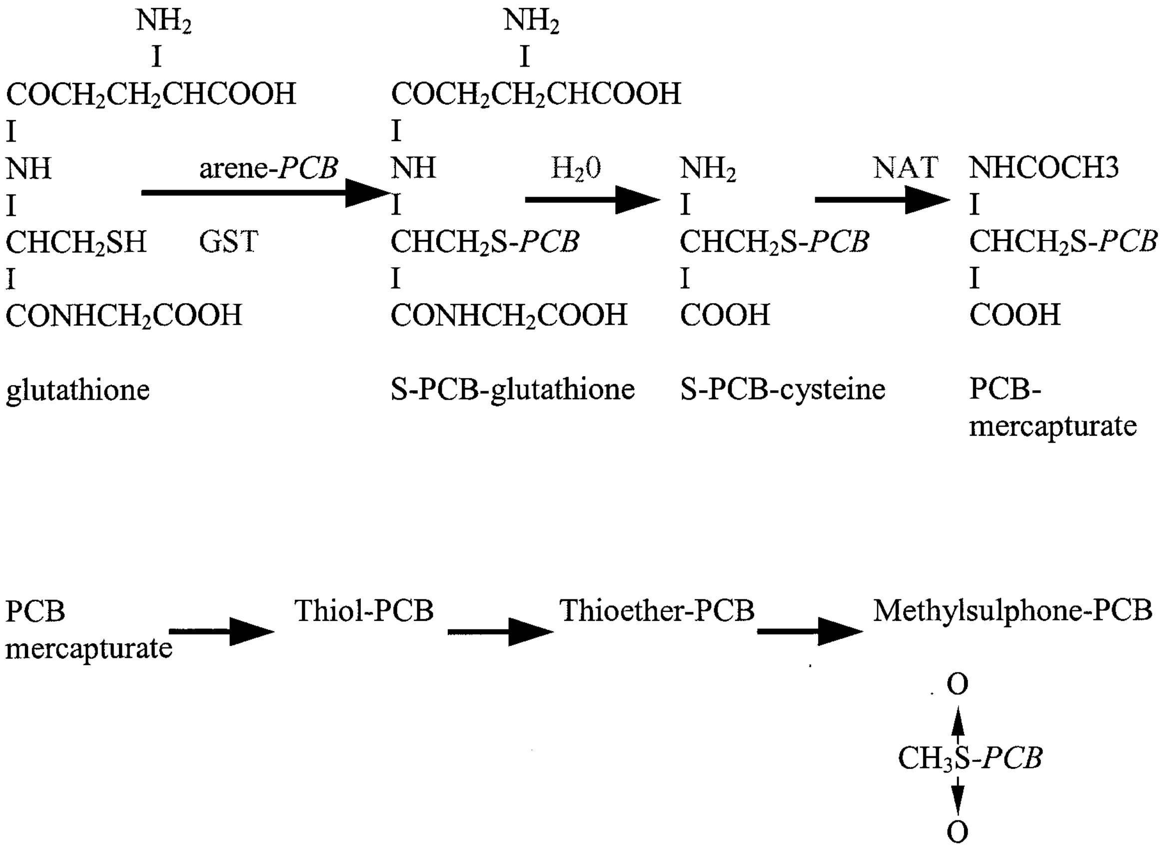
Glycosylation

In animal species, –OH, -NH, -SH, and –COOH groups can be substituted with glucuronic acid (GA) from uridine di-phosphate-GA by glucuronosyl transferase. As with most phase II reactions the purpose of this reaction is to increase the solubility of the xenobiotic and facilitate its removal from the organism.

5.1.4 Regulation of Metabolism

Many PCBs induce the production of a range of metabolic enzymes including several involved in the breakdown of a range of xenobiotics [462]. The commercial mixture Aroclor 1254 stimulates increased expression of phase I and phase II metabolic enzymes in the livers of rats and humans [463] specifically phase I enzymes CYPs 1A1, 1A2, 2B1, 2B2, 2A1 and 3A1 and phase II enzymes epoxide hydrolase, glucuronosyl transferase, glutathone-S-transferase, and various reductases [464]. Synthesis of CYPs 1A1 and 1A2 is known to be induced by 3-methylcholanthrene or β-napthoflavone, pharmacological arylhydrocarbon receptor (AhR) ligands, while CYPs 2B1, 2B2 and 3A1 are expressed in response to phenobarbital [465]. CYP 3A1 is also induced by glucocorticoid receptor ligands [466, 467].

Further elucidation of gene transcriptional pathways has identified certain subsets of congeners as being able to induce specific CYP expression. For example, co-planar PCBs are able to bind to the AhR and influence the expression of the genes it regulates [468]. Similarly, only very highly chlorinated PCBs induce CYP 3A1 expression [469, 470]. Although the ability of congeners to interact directly with the glucocorticoid pathway has not been demonstrated, it has been shown that the methylsulphone derivative of 2,2',4',5,5',6-hexachlorobiphenyl (PCB149) is able to bind the glucocorticoid receptor [471].



$$\begin{array}{c}\mathrm{NH_2}\\|\\\mathrm{COCH_2CH_2CHCOOH}\\|\\\mathrm{NH}\\|\\\mathrm{CHCH_2SH}\\|\\\mathrm{CONHCH_2COOH}\end{array}\xrightarrow[\text{GST}]{\text{arene-}PCB}\begin{array}{c}\mathrm{NH_2}\\|\\\mathrm{COCH_2CH_2CHCOOH}\\|\\\mathrm{NH}\\|\\\mathrm{CHCH_2S\text{-}}PCB\\|\\\mathrm{CONHCH_2COOH}\end{array}\xrightarrow{\mathrm{H_20}}\begin{array}{c}\mathrm{NH_2}\\|\\\mathrm{CHCH_2S\text{-}}PCB\\|\\\mathrm{COOH}\end{array}\xrightarrow{\text{NAT}}\begin{array}{c}\mathrm{NHCOCH3}\\|\\\mathrm{CHCH_2S\text{-}}PCB\\|\\\mathrm{COOH}\end{array}$$

$$\begin{array}{c}\mathrm{O}\\\uparrow\\\mathrm{CH_3S\text{-}}PCB\\\downarrow\\\mathrm{O}\end{array}$$

Figure 5-3: Mercapturation – methyl sulphone formation.
GST = glutathione S-transferase; NAT = N-acetyl transferase. From Crosby *et al.* [450].

5.1.5 Exposure and Accumulation

The estimated daily North American adult PCB intake in 1982-1984 was 0.5 ng/kg/day, mostly from meat and dairy products [472]. The offspring of women exposed to PCBs experience exposure both *in utero* and through breast feeding. Even though PCBs are detected in human tissues and breast milk at levels which are considered low regarding most toxicological endpoints, these concentrations have been reported to be harmful to reproductive, developmental, and endocrine processes [473, 474]. The average PCB concentration in whole human breast milk ranges from 10 to 180 ng/mL, which leads to a range of 1.5 to 27 μg/kg/day of PCBs that breast fed infants may consume [475]. Infants and young children consume more food per body weight than adults, and consequently experience a greater relative exposure to PCBs than adults. Furthermore, those in developmental stages where hormone levels play a critical role may have increased susceptibility to any perturbation of endocrine homeostasis. A study which correlated the concentration of PCBs in maternal serum (4.7 ng/mL) to that in cord serum (2 ng/mL) seems to demonstrate that PCBs can cross the placenta [476].

Individuals exposed to PCBs by occupational exposure or ingestion of highly contaminated foods can have much higher body burdens of PCBs. Capacitor plant workers in the United States have reported serum PCB levels ranging from 1 up to 1700 ng/mL [236]. People whose diet is rich in Great Lakes fish have serum PCB levels ranging from 7 to 366 ng/mL, depending on the amount of fish they consumed [236, 477]. Individuals exposed to PCBs by eating PCB-contaminated rice oil in Japan in the late 1960s developed a condition termed Yusho. A similar case of PCB poisoning occurred in Taiwan in 1979, which was termed Yu-Cheng, in which those affected displayed the same symptoms seen in Japan. Yusho and Yu-Cheng patients had levels of PCBs reported in the 50 ng/mL range as well as polychlorinated dibenzofurans (PCDFs) at levels around 0.1 ng/mL in their blood [478]. Children of exposed Yusho and Yu-Cheng mothers developed distinct clinical manifestations including dark brown skin and mucous membrane pigmentation, edematous eye, dentition at birth, abnormal skull calcification, and low birth weight [479]. The effects seen in these children demonstrate that organochlorines such as PCBs and polychlorinated dibenzofurans (PCDFs) are potent teratogens.

The Inuit population is exposed to large amounts of PCBs through their diet rich in the marine mammals which are near the top of the Arctic aquatic food chain [258]. These animals themselves have bioaccumulated levels of PCBs as high as 8μg/g of lipid, which are stored at highest levels in the blubber at 1.9 μg/g (wet weight) and are also detected in other tissues consumed as meat at 0.8 μg/g [236, 468]. Inuit women in northern Québec have approximately 7 μg/g of lipid of PCBs in their breast milk, which is 7 fold higher than that found in Caucasian women in southern Québec [258].

5.1.6 Health Effects of PCBs

The levels of PCBs detected in some human tissues and breast milk have been shown to produce health effects including developmental, reproductive, endocrine, and cancer related outcomes. A wide range of toxic and teratogenic effects attributed to PCB exposure has been observed in experimental animals, as well as wildlife and humans. Individuals exposed to high levels of PCBs in the workplace or through contaminated food show changes in hormone levels, chloracne, liver damage, and the infants of exposed mothers exhibit developmental defects [236]. This wide range of health effects is the product of a number of variables: the type of PCB; the amount of PCB in the exposure; the length of exposure; the developmental time point at which the exposure occurred; and the organism exposed. Although PCBs have relatively high LD_{50}s at approximately 1300 mg/kg (depending on the composition of the PCB mixture), toxic effects are seen at exposures orders of magnitude lower. PCB toxicity studies have been performed in various organisms including mice, rats, mink, guinea pigs, and monkeys. In general mink and monkeys exhibit a higher degree of sensitivity to PCBs than other species, particularly in regard to changes in body weight [480, 481]. Variation of PCB effects in different species may be modulated by species-specific discrepancies in metabolism, enzyme induction and quantitative differences in PCB binding to receptors in target organs [482]. The toxicity of PCBs in humans has been measured in individuals exposed through occupational or accidental exposure.

The immune system has been shown to be susceptible to the effects of PCBs. Serum immunoglobulin levels decreased and susceptibility to microbial infections increased in mice and rats treated with PCBs for 3 to 6 weeks [483-486]. PCBs have also been shown to cause similar effects in monkeys [487-489]. The effects of PCB exposure on the human immune system are unclear. Although the Yucheng and Yusho children show symptoms which indicate immune suppression (higher incidence of microbial infection) biochemical markers indicate they have normal immune function [490-492].

Neurological effects of PCBs have been reported in humans and animals. Individuals with high dietary exposure such as the Inuit and those consuming fish from the Great Lakes have been studied in depth to monitor these effects. PCB exposure has been correlated to changes in neuropsychological functioning in groups of people with a diet rich in fish [493]. Studies of PCB effects at different stages of development in humans and rodents show alterations in dopamine levels [236]. PCBs can also affect memory, attention, and learning in humans and monkeys exposed *in utero* and through lactation [494, 495].

In addition to the induction of metabolic enzymes, PCBs appear to have general histopathological effects on the liver. PCB doses above 0.3 mg/kg/day for 3 to 15 weeks have been shown to cause increased liver weights in rats [496-500] although biochemical alterations were not observed in all of these studies. The lowest observed adverse effect level for PCB induced hepatic effects in mice is 200 mg/kg, and the no observed adverse effect level is a single dose of 50 mg/kg. Histological effects of PCBs in rats are seen at doses of 50 mg/kg/day for 30 days and 11 months and of 5 mg/kg/day for 6 months. The effects observed in these studies include hepatocyte hypertrophy, fat deposition, fibrosis, necrosis, and changes in serum levels of liver associated enzymes indicative of possible hepatocellular damage [236].

In many animal studies, changes in body weight were associated with PCB exposure. Reduced body weight after a single high dose exposure was attributed to dehydration [501], however intermediate and chronic dietary administration of PCBs often cause a decrease in body weight which is considered to constitute a wasting syndrome [236].

5.1.7 PCBs and Cancer

Among case-control studies of non-Hodgkin's lymphoma, one study found an association with both 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-like and several non-TCDD-like congeners [502], while another found a multiplicative interaction with seropositivity for the Epstein-Barr virus early antigen [503]. Since PCBs suppress the immune system and immunosuppression is an established risk factor for non-Hodgkin's lymphoma, this may also contribute to the risk of PCB-induced cancer.

Many epidemiological studies on breast cancer and environmental exposure to PCBs have been conducted. Results from *in vitro* studies on the estrogenicity of PCBs suggest that these compounds may be contributing factors to high rates of breast cancer in industrialized countries. However, the relationship between PCBs and breast cancer remains unclear. Some studies show a significant correlation between breast adipose tissue levels of PCB congeners and breast cancer risk [183]. It has been suggested that the ability of PCBs to produce free radical-mediated oxidative DNA damage during oxidation of lower chlorinated biphenyls may induce carcinogensis [504]. The detection of increased oxidative damage of DNA in human breast tumour tissue suggests that this hypothesis warrants further investigation. Another study found that PCBs are present in breast cyst fluids and not just adipose tissue and therefore come into contact with the breast ductal epithelium [505]. Despite this evidence, other epidemiological studies have found no correlation between serum PCBs and breast cancer risk [506-508].

5.1.8 Endocrine-disrupting Characteristics of PCBs

It is perhaps no surprise that research has indicated that, as with metabolism and accumulation, the toxicity of PCBs is determined by their individual structures. Some congeners seem to be relatively harmless while a few are highly toxic [468]. The toxicity is dependent in part upon the species exposed; some congeners are toxic to rats and yet apparently benign in monkeys. In addition, there is evidence that toxicity can be mediated by a variety of mechanisms; therefore, there is no unifying structural feature that identifies congeners as potentially toxic.

Planar and Co-planar PCBs Interact with AhR

Studies of the mechanism of toxicity of PCBs have demonstrated that a number of congeners are able to elicit the same toxic and cellular responses as TCDD and related halogenated aromatic hydrocarbons [468, 509]. TCDD toxicity is almost exclusively mediated through the ability to bind to the arylhydrocarbon receptor (AhR) and regulate gene transcription [510]. Amongst the genes known to be up regulated by the binding of liganded AhR to the specific DNA recognition site (xenobiotic response elements) in promoter regions are those encoding cytochromes P450 1A1 and 1A2 and other metabolism genes [511-513].

The key structural feature of PCDD-like PCBs is a co-planar structure that requires the absence of ortho-chloro substituents (positions 2 or 6). TCDD-like activity is optimised by substitution at both para- positions, and two or more meta- substitutions. PCBs such as 3,3',4,4',5-pentachlorobiphenyl, and 3,3',4,4',5,5'-hexachlorobiphenyl have been shown to compete well with ^{3}H-TCDD for binding to the AhR [514, 515]. Mono-ortho-PCBs and even selected di-ortho-PCBs have also been demonstrated to activate TCDD-like pathways in cells and at high doses. However, in competitive binding assays these PCBs have been found to have much lower affinities for the AhR, and do not present a significant toxic threat via this route at physiological concentrations [468].

Other Hormonal Routes

Besides TCDD mimicry, PCBs bind several other endocrine receptors and thereby potentially disrupt normal endocrine function. The same properties that make certain PCB congeners good ligands for the AhR may also endow them with thyroid hormone-like potential. Studies of the structure-activity relationships of thyroids and the proteins to which they bind indicate that they fall into two groups. For those that bind T3 in preference to T4, such as the nuclear thyroid hormone receptor, the structure of the inner (tyrosyl) ring is most important. A constrained, planar inner ring such as that seen for AhR ligands correlates well with functional activity (receptor binding). For proteins that bind T4 in preference to T3, including the major serum thyroid hormone transporters (globulin, prealbumin and albumin) the structure of the outer (phenolic) ring is of greater importance. Consequently many ortho-substituted PCBs are able to bind these proteins although lateral chlorine substituents alone still yield optimal binding [516, 517]. Since almost all persistent PCBs are laterally substituted, it can be anticipated that most of the PCBs in animal tissues are able to bind thyroid hormone transport proteins.

Exposure to PCBs in experimental animals *in utero* and/or during early development (e.g. through breast milk) may give rise to a hypothyroid state during development. Studies of rats treated with two PCDD-like congeners at 0.2 to 1.8 mg/kg [518] and 5 to 50 mg/kg/day for 5 to 7 months [519] found that serum and plasma T3 and T4 levels were suppressed in a dose-related manner in neonatal, pregnant, and adult rats.

Thyroid hormones play an important role in normal brain development, as is evident from neurodevelopmental disorders and deficits associated with hypothyroidism, including auditory, motor, and intellectual deficits. These outcomes underscore the importance of thyroid hormones in the normal development of the fetal cochlea, basal ganglia, and cerebral cortex, which begin to develop in humans during the second trimester of gestation. This is also the time period during which the fetal thyroid gland becomes functional. PCBs have been shown to reversibly increase Sertoli cell proliferation and testis weight through suppression of T4 in rats treated with 1.6 and 3.2 mg/day during gestation [520].

In addition to inducing hypothyroidism, 50 and 500 mg/kg of PCBs administered to rats translactationally as well as directly via the oral route produced ultrastructural lesions such as an increased development of rough endoplasmic reticulum and mitochondrial vacuolization in thyroid follicular cells. This contributed to decreases in serum thyroid hormone levels [521, 522]. The decrease in serum thyroxine levels in this case is attributed to an interference with hormone secretion and an enhanced peripheral metabolism of thyroxine [523].

Although evidence linking PCB exposures to thyroid morbidity in humans is limited, the risk for goiter was significantly increased among the Yucheng cohort [524], indicating the possibility of excess thyroid disease in an adult population that experienced relatively high exposures to mixtures of PCBs and PCDFs. Studies of the relationships between PCB exposure and thyroid hormone status in children or adults have reported a variety of different results, with findings of both positive and negative correlations between PCB exposure and circulating levels of T3, T4 or thyroid stimulating hormone (TSH).

The most compelling evidence for a potential thyroid hormone involvement in PCB toxicity in humans is based on observations made in experimental animals, including rodents and nonhuman primates. Major findings include (1) histological changes in the thyroid gland indicative of both stimulation of the gland (similar to that induced by TSH or a hypothyroid state) and a disruption of the processing of follicular colloid needed for normal production and secretion thyroid hormone [525]; (2) depression of T4 and T3 hormone levels, which may effectively create a hypothyroid state; (3) increased rates of elimination of T4 and T3 from serum; (4) increased activities of T4-UDP-glucuronyl transferase in liver, which is an important metabolic elimination pathway for T4 and T3; (5) decreased activity of iodothyronine sulfotransferases in liver, which are also important in the metabolic elimination of iodothyronines; (6) decreased activity of iodothyronine deiodinases including brain Type-2 deiodinase, which provide the major pathways for the production of the active thyroid hormone, T3; and (7) decreased binding of T4 to transthyretin, which is an important transport protein for both T4 and T3. These observations indicate that PCBs can disrupt the production of thyroid hormones, both in the thyroid and in peripheral tissues, can interfere with their transport to peripheral tissues, and can accelerate the metabolic clearance of thyroid hormones.

PCBs are able to suppress retinoid levels in serum and other tissues [526]. Due to the intimate relationship between thyroid and retinoid pathways it is possible that these effects are a result of the impact of PCBs on thyroid pathways [527].

Interactions with Steroid Hormone Receptors

Hydroxylated PCBs have been found to bind specifically to ERα in mice [528]. Metabolically para-hydroxylated PCBs that are conformationally restricted due to ortho-chlorine substitution appear to be the most effective at binding ER [527]. The hydroxyl group may aid in steroid mimicry due to its ability to form hydrogen bonds with the receptor ligand-binding site similar to those made by the endogenous ligand. Overall, lipophilic properties of the PCB congeners appear to be important to their ability to bind ER. Our work [200, 529] on PCBs in androgen and glucocorticoid receptor systems indicates that certain congeners are also able to specifically interact with other steroid receptors both *in vitro* and *in vivo*.

Other research has associated both PCDD-like and non PCDD-like congeners with toxicity due to endocrine disruption [530]. PCB exposure may lead to disruption of endocrine homeostasis as a result of the ability of many congeners to induce the expression of proteins involved in hormone metabolism. Several of the enzymes induced (e.g. CYP 1A2 & 3A1) and the pathways that are activated are involved in steroid and thyroid hormones metabolism [531, 532]. Increased expression of these enzymes could lead to chronic suppression of the overall levels of several hormones in an individual. A decline in key hormone levels frequently stimulates an increase in their production. Up regulation in steroid synthesis may result in some tissues, particularly near the site of synthesis, becoming overexposed to the hormones' proliferative effects. In some cases, this can cause those tissues to develop insensitivity to the hormones, for example via down-regulation of the corresponding hormone receptor protein.

In vitro Evidence

Using the E-Screen assay, five PCB congeners as well as several hydroxylated PCB metabolites were found to be estrogenic [402]. Further studies examined the binding affinity of hydroxylated PCB congeners and found the most potent congener was 2,4,6,2',6'-pentachloro-4-biphenylol, which binds the ER with a IC_{50} of 70 nM, approximately 5 times less potent than 17ß-estradiol [533, 534]. The congeners found to be estrogenic were not coplanar. The degree of planarity is determined by the presence of ortho-substituted chlorines, which cause steric hindrance to rotation. Coplanar PCBs have at most a single ortho-substituted chlorine atom.

In vivo Evidence

Studies of endocrine disruption by PCBs have examined a number of hormonal systems and have been conducted in animals and in humans through epidemiological analysis. A study was conducted on the effects of PCBs on turtles that utilize temperature-dependent sex determination (common in many egg-laying reptiles). The investigators found that in turtle eggs incubated at male-producing temperatures, administration of PCBs reversed the gonadal sex to female [535].

Evidence of decreased serum levels of adrenal cortex hormones has also been reported in animal studies. Alterations in adrenal function including decreased serum corticosterone levels in rats treated orally with 1 to 50 mg/kg/day PCBs for at least 5 months has been reported [536]. By contrast, an increase in serum corticosterone levels after treatment with at least 8.1 mg/kg/day of PCBs for 2 weeks has been observed in mice [537]. However, a study of monkeys exposed to 0.08 mg/kg/day of PCBs for 22 months showed no change in serum hydrocortisone [538]. These discrepancies may be due to species-specific differences in glucocorticoids and the effects of PCBs on the metabolism of glucocorticoids in each species [236].

PCBs were uterotrophic when administered to immature female rats in doses of 30 and 120 mg/kg [539]. PCBs at 0.1 to 10 μg/ml also decreased the *in vitro* fertilizing ability of exposed mouse oocytes [540]. Monkeys treated with 20 and 80 μg/kg/day PCBs for two years experienced changes in luteal phase progesterone levels and a marginally longer duration of menses [541]. Neonatal female rats exposed to 110 μmol/kg PCBs exhibited increased hepatic basal testosterone hydroxylase activity, androstenedione formation, and testosterone metabolism [542]. Female offspring of rats exposed to PCBs as low as 8 μg/kg during lactation underwent a delay in puberty. When treated with doses between 32 and 64 μg/kg these rats had decreased uterine wet weight, and offspring in the 64 μg/kg group exhibited impaired fertility and irregular estrous cycle patterns [543].

Endocrine modulating effects related to estrogen have also been observed in humans. Women who consumed PCB contaminated fish had a significant reduction in menstrual cycle length [544]. Another study found high levels, around 790 ng/mL, of some PCB congeners in the blood of women with repeated miscarriages which correlated with immunological and hormonal changes, such as a decrease in testosterone levels [545].

Exposure of sperm to a capacitation medium containing PCBs did not effect *in vitro* fertilizability or sperm motility in mice [540]. However, male rats treated with 100 μmol/kg PCBs neonatally exhibited decreased hepatic basal testosterone hydroxylase activity and androstenedione formation [542]. The male offspring of rats dosed with 8, 32, and 64 mg/kg/day PCBs for 6 days during lactation developed significantly smaller prostates with fewer acini and altered morphology of the epithelial cells compared to controls in adulthood [546]. Studies have also shown rats treated during lactation and/or *in utero* developed larger testes [520, 546]. A reduction in seminal vesicle and epididymal weights, and caudal epididymal sperm counts has been reported [473] in rats treated during development. These studies indicate some of the potential reproductive target sites of PCBs in males; however, these findings are not consistent with other reports [473, 547]. The effects of PCBs on male reproductive organs such as the prostate, epididymis, and testis are examined in this study.

5.2 Aroclor Results

5.2.1 Aroclor Results *in vitro*

Aroclor 1254 has a variety of systemic toxic effects in animal models, including anaemia, reversibly increased liver weight, and alterations in liver enzyme functions, with variable histological findings. This mixture has also been shown to cause a significant decrease in seminal vesicle and epididymal weights in rats exposed for 5 weeks post-weaning, and a decrease in cauda epididymal sperm counts at 15 weeks after weaning [547].

Since Aroclor 1254 is 54% chlorine by weight, pentachlorobiphenyls represent the median congener configuration. From published analysis, 77 congeners in total are found above detection threshold levels although the precise composition of the mixture varies slightly between batches [236].

AR Mediated Luciferase Transactivation

Aroclor 1254 was tested in two cell lines (PC-3 and LNCaP) for its effect upon AR mediated transcription of the firefly luciferase gene. Significant antagonistic effects were seen versus DHT in LNCaP cells at Aroclor concentrations above 100 nM and 50% suppression was produced by 700 nM Aroclor 1254 (Figure 5-4). PC-3 cells produced a less clear pattern where only Aroclor 1254 produced a significant effect (Figure 5-4A).

The same tests were performed with Aroclor 1260 and the results in LNCaP cells were similar, although 1260 appears to be a less potent antagonist than 1254 only producing a statistically significant effect at the highest concentration tested (10 μM) (Figure 5-4). In PC-3 cells however 1260 consistently failed to exhibit any activity either agonist or antagonist (Figure 5-4A). Two further Aroclor mixtures, 1242 and 1248, were also tested in LNCaP and were both found to have equivalent or greater antagonist activity to Aroclor 1254 (Figure 5-4B and C). In experiments without DHT, only Aroclor 1254 produced significant induction of firefly luciferase (Figure 5-5) at concentrations tested, up to 10 μM.

To investigate the underlying mechanism of Aroclor transcriptional interference several different competitive binding assays were undertaken to determine whether Aroclor components could displace DHT from its cellular binding sites (Figure 5-6). In the first method, PC-3 cells transfected as for the firefly luciferase transactivation assay were treated with radiolabeled DHT in combination with Aroclor 1254. After a 20-hour incubation time cells were harvested and washed and the cellular fraction of the radioactivity used as a measure of ligand displacement by the PCB mixture. It was found that Aroclor 1254 was able to displace radiolabeled DHT from the cells (Figure 5-6A). To further investigate the role of the androgen receptor in this displacement HeLa cells stably transfected with AR were treated with tritiated DHT and Aroclors were added as cold competitors. Aroclor 1254 proved to be an effective competitor for DHT binding to AR, in a dose dependent manner (Figure 5-6B).

Finally, to determine if there is a specific direct interaction of Aroclor PCBs with the AR ligand-binding domain (AR-LBD) purified recombinant protein (PanVera) was mixed with radiolabeled ligand and Aroclor and the ability of each mixture to compete out agonist binding to the AR was measured (Figure 5-6C). It was found that the four Aroclors can bind the AR-LBD and displace classical ligands with potencies in the order 1242>1248>1254>1260.

Affects of Aroclors on GR and ER

Aroclors 1254 and 1260 were added to PC-3 and HepG2 cells transiently transfected with GR and ER expression plasmids, respectively, together with reporter constructs encoding the firefly luciferase gene under the control of a corresponding specifically regulated promoter. No significant effects were seen with either mixture upon the ability of either receptor to activate transcription (Figure 5-7 and Figure 5-8). Aroclor 1254 was also tested in PC-3 cells for its ability to displace radiolabeled DEX from cellular binding sites (Figure 5-6A) but was found to have no significant effect.

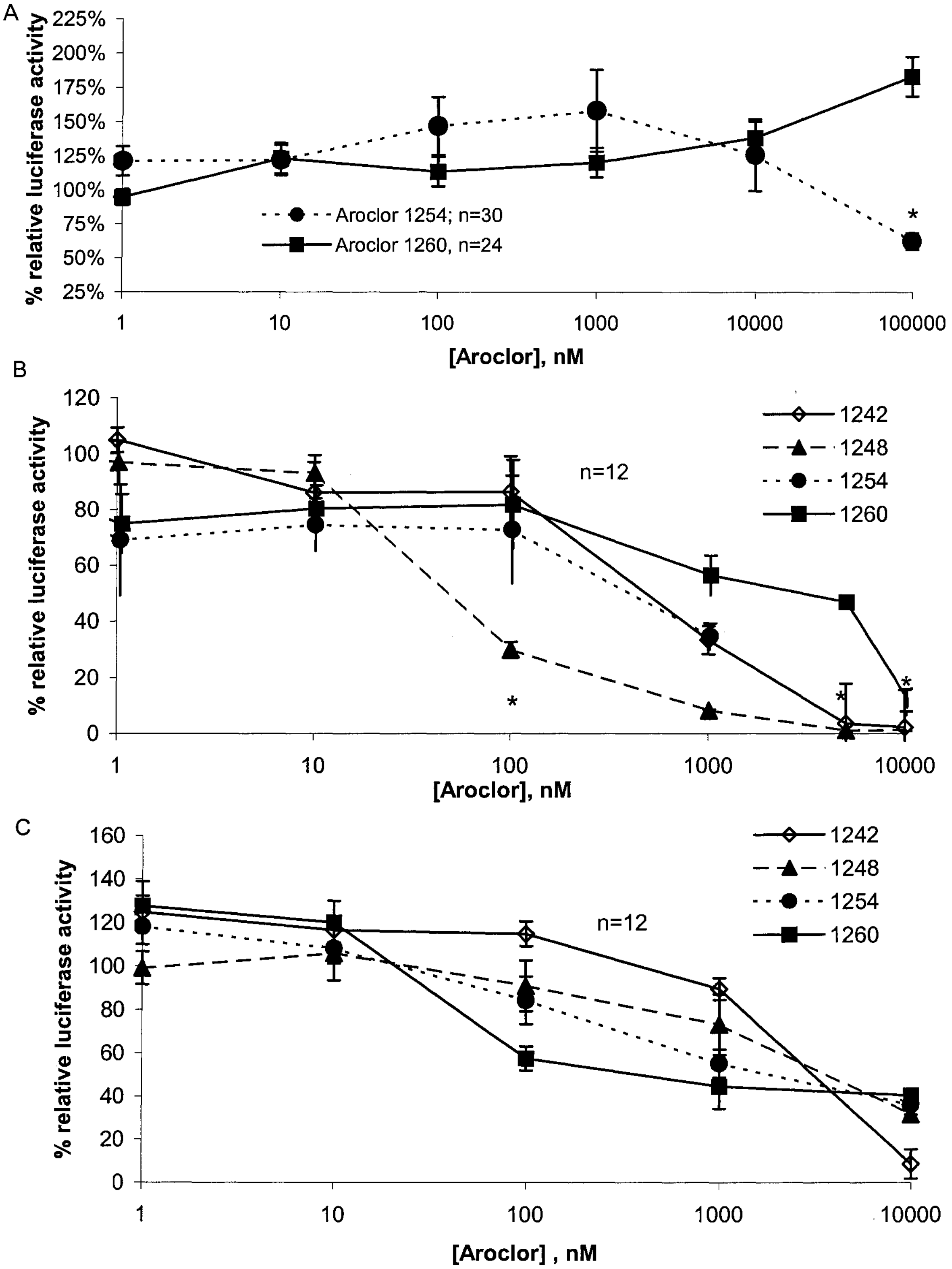


Figure 5-4 :The effects of four Aroclor mixtures on androgen driven transcription in transiently transfected PC-3 cells (A) and in LNCaP cells with 0.05nM DHT (B), and 1.0nM DHT (C).
The * indicates a value is significantly different ($p<0.05$, using Student's T-test) from control (no Aroclor added, set at 100% relative luciferase activity) . Number of individual wells (n=), from multiple experiments, used to derive the graph is shown.
Error bars indicate the standard error of the mean (SEM).

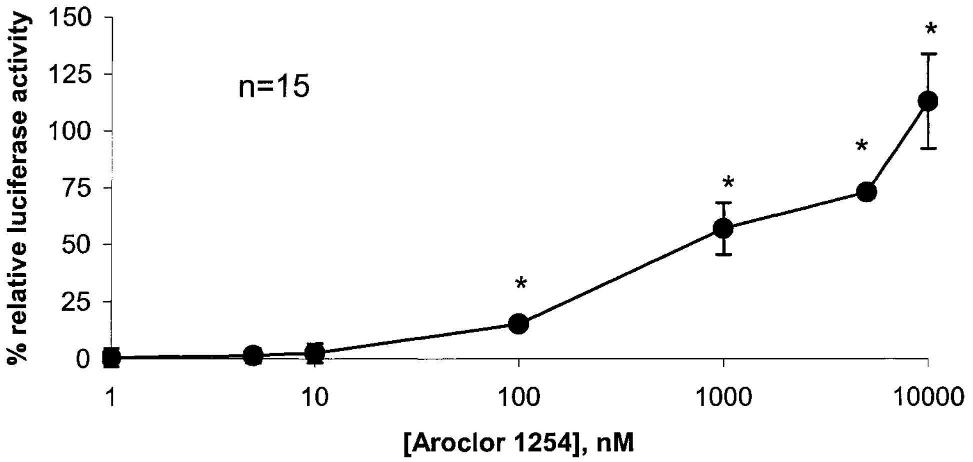


Figure 5-5: The effects of Aroclor 1254 on androgen driven gene transcription without DHT. Aroclor 1254 was applied to AR transfected LNCaP cells and resultant luciferase expression was measured. The * indicates a value is significantly different ($p<0.05$, using Student's T-test) from control (no Aroclor added, set at 100% relative luciferase activity). Number of individual wells (n=), from multiple experiments, used to derive the graph is shown. Error bars indicate the standard error of the mean (SEM).

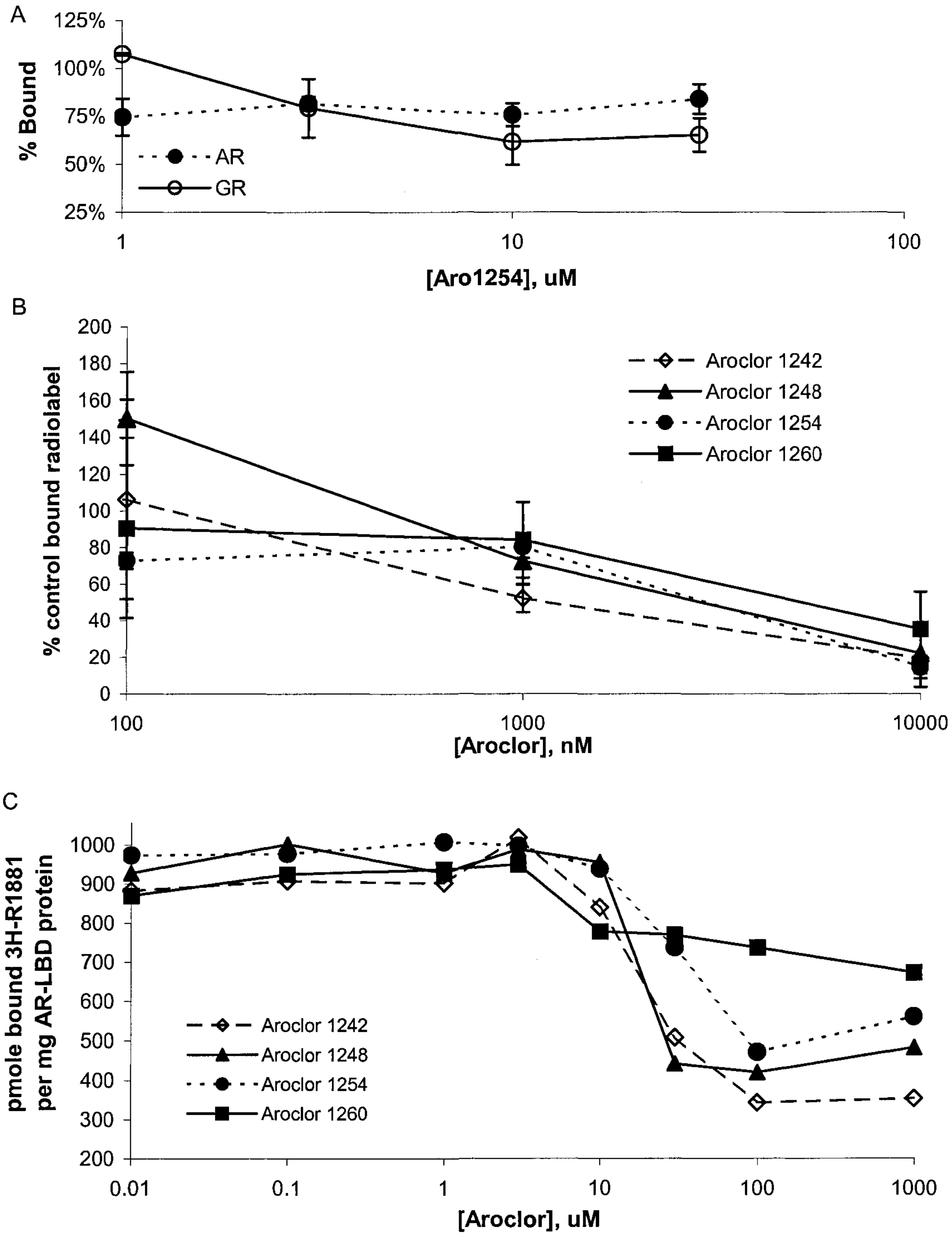


Figure 5-6: The ability of Aroclors to compete with known agonists for the ligand binding site on steroid receptors. Aroclor 1254 versus radiolabelled DHT and DEX in AR and GR transfected PC-3 cells respectively (A). Four aroclors displace ^{3}H-DHT from AR-HeLa cells (B), and compete with ^{3}H-R1881 for binding to purified recombinant AR-LBD (C). The * indicates a value is significantly different ($p<0.05$, using Student's T-test) from control (no aroclor added; set at 100%). Error bars indicate the standard error of the mean (SEM)

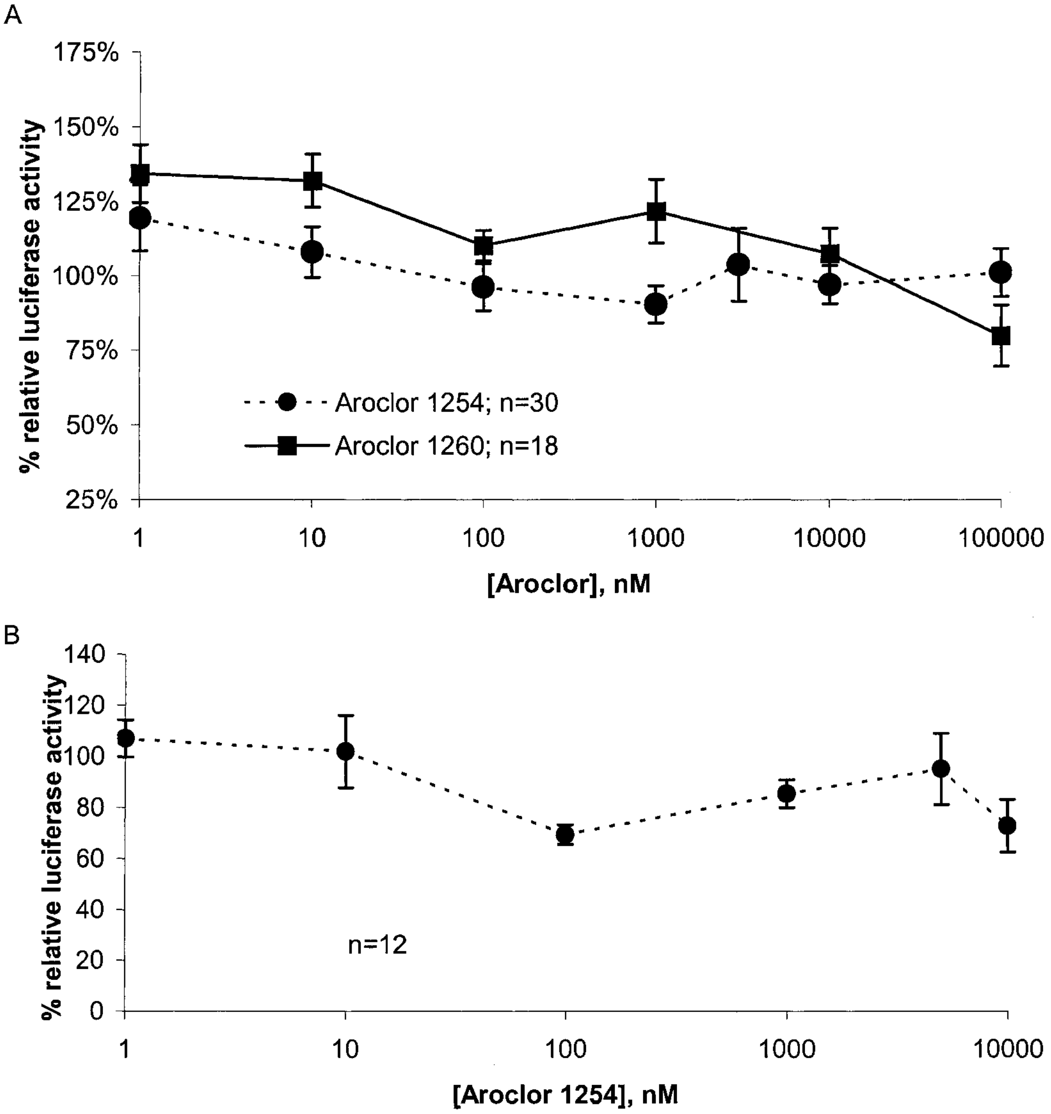


Figure 5-7 : The effects of Aroclors on GR mediated transactivation. With 1nM DEX in transiently transfected PC-3 cells(A), and LNCaP cells (B).

Number of individual wells (n=), from multiple experiments, used to derive the graph is shown. Error bars indicate the standard error of the mean (SEM).

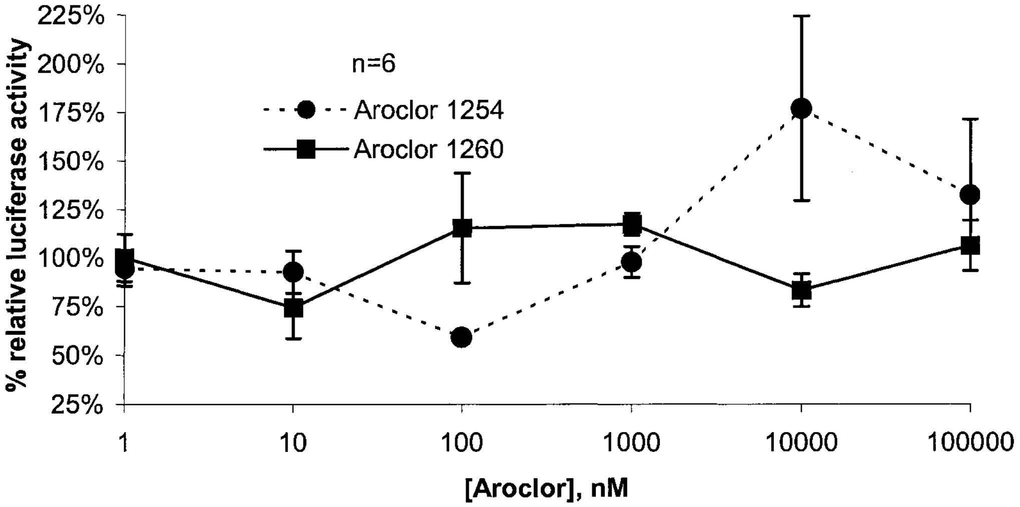


Figure 5-8 : The effects of two Aroclors on estrogen driven gene expression. Luciferase transactivation in HepG2 cells transiently transfected with ER. Number of individual wells (n=), from multiple experiments, used to derive the graph is shown. Error bars indicate the standard error of the mean (SEM).

5.2.2 Aroclor 1254 in LPB-CAT Mice

The *in vitro* cell culture studies demonstrated that PCBs have properties of potential endocrine disruptors. All Aroclors tested appear able to compete with natural ligand for binding to AR; Aroclor 1254, at least, can induce reporter gene activity through the AR. Given these results, as well as reports of the ability of Aroclors to alter steroid hormone systems and reproductive development, an examination of *in vivo* effects of Aroclor 1254 was undertaken by Cheryl Portigal.

The effects of Aroclor 1254 on the weights and histology of selected organs was investigated and prostate development in particular was examined by measuring the expression of the androgen-driven prostate specific LPB-CAT transgene. The organs examined were androgen-target and reproduction related organs (testes, epididymis, and prostate) as well as liver, an established target for PCB effects as well as the site of steroid hormone metabolism.

PCBs are known to bioaccumulate and are stored preferentially in adipose tissue, but are also present in serum, blood plasma, and milk [236]. Consequently, PCBs can be transferred to offspring via the placenta and through breast milk. The offspring of exposed mothers, who are exposed to PCBs at sensitive developmental stages, will likely be more susceptible to the effects of PCBs than those exposed during adulthood.

Two studies were undertaken to evaluate the effects of Aroclor 1254 exposure during development. These studies were designed to look at chronic exposure to PCBs through different stages of development, rather than acute exposure that is tested in many toxicology studies. In the single dose level study, dams were dosed with 10 mg/kg/day from the time of mating until the end of the lactation period at three weeks. Weanling mice were then administered 10 mg/kg/day Aroclor 1254 until four weeks of age (prepubertal) or eight weeks of age (post pubertal), in order to assess the effects of Aroclor 1254 at different stages of development. In the dose response study, dams were treated with 10, 20 or 40 mg/kg/day Aroclor 1254 from the time of mating, until the end of the lactation period at three weeks. Weanling mice were then administered the corresponding dose until eight weeks of age, to test the effects of different doses of Aroclor 1254.

Alteration of Organ Weights

Body weights of mothers being dosed, suckling pups, and weanlings were collected throughout both studies. Due to litter-to-litter differences, the treatment mice in the single dose level group were heavier than the control group. An increase in body weight is not characteristic of PCB exposure, and in fact a decrease in body weight has been reported as a health effect of these compounds [236]. The differences in body weight in the mice in this study may be attributable to litter size. Some of the treatment mice were from smaller litters, which can yield larger pups. However, there was no trend in litter size observed when treatment and control groups were compared. This pattern was not seen in the dose response group, where there were no measurable differences seen between dose groups. To correct for the variation in body weight between individual animals in this study, organ weight was corrected for each animal's body weight.

Liver

To determine if there was an effect on the liver, a known target organ of PCBs, the livers of treated mice were examined for changes in weight, although the doses used were below levels shown to cause overt effects in other studies [548]. There was a significant difference in liver weight/body weight between control and treatment mice in the single dose level study at four weeks of age (Table 5-2), however, liver weight/body weight in the dose response treatment mice were significantly higher for the 20 and 40 mg/kg/day dose groups than controls (Table 5-3). This is consistent with reports in the literature of effects of PCB on the liver [473, 496, 497, 499, 500]. An increase in liver weights due to PCB exposure is usually associated with hepatocyte enlargement, which is not considered an adverse effect, unless certain biochemical changes along with notable histological alterations are also observed [236].

	Organ	Corrected organ weight (g)	
Mice at Four Weeks		CONTROL (N=12)	TREATMENT (N=5)
	Prostate	2.99±0.20	2.7±0.25
	Epididymis	7.69±0.26	7.2±0.22
	Testis	27.15±1.38	28.4±0.70
	Heart	51.84±0.94	49.16±3.3
	Liver	537.42±5.25	625.66±11.49*
	Kidney	69.12±1.76	65.16±1.98

	Organ	Corrected organ weight (g)	
Mice at Eight Weeks		CONTROL (N=11)	TREATMENT (N=8)
	Prostate	4.38±0.27	3.99±0.29
	Epididymis	13.85±0.28	13.16±0.70
	Testis	42.17±2.12	43.87±1.81
	Heart	50.58±1.40	46.61±2.14
	Liver	494.10±15.00	540.05±21.88
	Kidney	72.38±2.25	72.86±3.21

Table 5-2: Organ weights from LPB-CAT mice treated with 10 mg/kg/day Aroclor 1254. Corrected organ weights for prostate, left epdidymis, left testis, heart, liver, and kidney (organ weight/body weight in grams/gram body weight x10^4) of LPB-CAT mice treated to four and eight weeks of age with vehicle only (canola oil) and 10 mg/kg/day Aroclor 1254. Values are mean ± SEM. *p<0.01 by Student's T-test.

	Control (n=9)	10 mg/kg/day (n=5)	20 mg/kg/day (n=11)	40 mg/kg/day (n=5)
Prostate	4.36±0.25	3.91±0.23	3.50±0.18*	3.20±0.27**
Epididymis	13.63±0.21	12.41±0.62	13.33±0.39	13.03±0.45
Testis	46.92±2.22	41.71±3.79	48.79±1.09	47.86±1.11
Heart	51.30±1.27	47.21±0.80	49.05±0.38	51.48±2.41
Liver	496.53±9.67	536.39±14.54	617.58±13.52**	718.08±24.26**
Kidney	74.72±2.44	75.63±2.59	75.67±1.70	78.30±2.72

Table 5-3: Organ weights from LPB-CAT mice treated with varying Aroclor 1254 dose. Corrected (organ weight/body weight in grams/gram body weight x10^4) for mice treated for eight weeks of age with canola oil, Aroclor 1254 at 10, 20, 40 mg/kg/day. Values are mean ± SEM. *p<0.05 ,**p<0.01 by Student's T-test.

Prostate

The prostates in the single dose level group did not show a significant difference in prostate weight to body weight ratio between treatment and controls for the single dose level group (Table 5-2). In the dose response study, prostate weight/body weight was altered by Aroclor 1254 treatment. Prostate weight/body weight for the 20 and 40 mg/kg/day dose groups were significantly lower than for the control group (Table 5-3).

CAT Activity - Single Dose Level Group

There was a reduction in CAT activity from the prostate extracts of the four and eight week treatment mice, which was statistically significant ($p<0.05$) in the eight week group (Figure 5-9A). These findings show antiandrogenic action of the Aroclor 1254 treatment on the specifically androgen responsive LPB-CAT reporter. Due to this effect at a relatively low dose of 10 mg/kg/day, we chose to look at mice at eight weeks of age, treated from conception in the same manner, but also using treatments 2 and 4 fold higher.

CAT Activity - Dose Response Group

The CAT enzyme activities in the dose response group showed a trend of a decrease in activity with increasing dose with the highest dose being statistically lower than the control ($P<0.05$ [Figure 5-9B]).

Serum Testosterone Levels

The level of testosterone in the serum may provide insight into the effects of antiandrogens on the endocrine system. This has been seen in studies of antiandrogen drugs used in prostate cancer treatment [549-551] as well as in the treatment of aggression in convicted male sexual offenders [552]. Patients treated with the antiandrogen flutamide develop increased serum testosterone levels since the antagonist inhibits the negative feedback effects of androgens on the hypothalamic-pituitary-testes axis [553]. Serum luteinising hormone (LH) increases in a dose related manner with flutamide administration [554] since androgen's primary negative feedback control is through regulation of gonadotropin secretion from the hypothalamus [555].

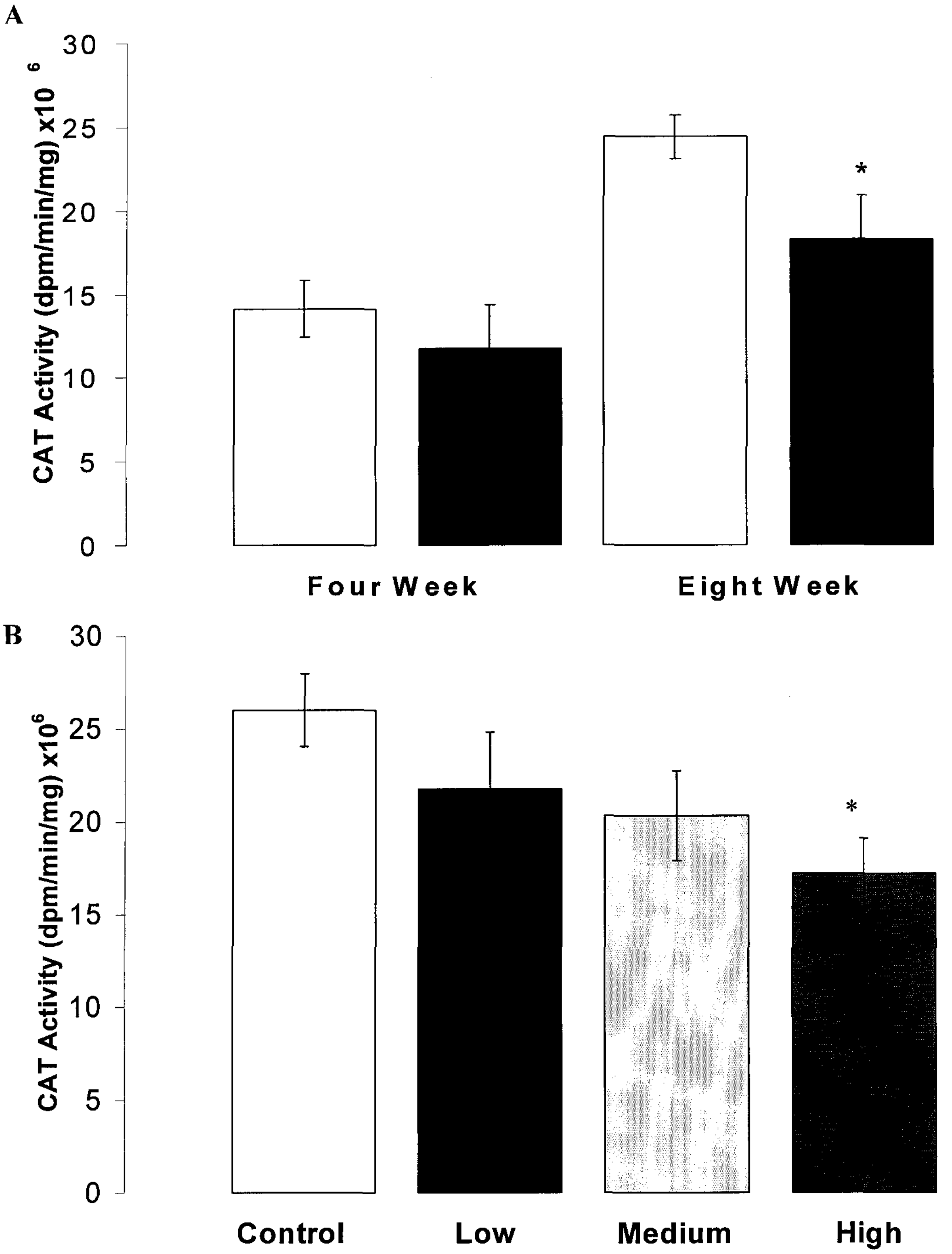


Figure 5-9: Activity of CAT reporter transgene in prostates of Aroclor 1254 dosed mice. [556] Prostate tissue extracts of four and eight week old control (open bars) and treatment (closed bars) LPB-CAT mice in the single dose level study. Mice were treated with 10 mg/kg/day Aroclor 1254 by gavage from conception until four or eight weeks of age (A). Prostate tissue extracts of eight-week-old LPB-CAT mice in the dose response study treated with vehicle only (control), 10, 20, and 40 mg/kg/day (B). Mice were treated with Aroclor 1254 by gavage from conception until eight weeks of age. Data is presented as mean CAT activity. Error bars represent SEM. *$p<0.05$, using Student's T-test.

There was a high degree of variation in testosterone levels within treatment groups, which is consistent with other studies in which the measured serum testosterone levels ranged from 0.7 mMol/L to 158 mMol/L in post pubertal mice [557]. Variations in serum testosterone levels in mice may be attributed to the release of LH from the pituitary in pulses [558, 559]. As testosterone synthesis is dependent on LH, these fluctuations may be expected and data was therefore presented as a scatter plot of testosterone concentrations (Figure 5-10). There were three serum testosterone levels that were much higher than the other fifteen samples measured. Statistical analysis of the data set was done both with and without these three points that may have skewed the results. ANOVA tests of the data with and without the higher testosterone levels both showed no significant difference between treatment groups ($p>0.05$). We also used the Kruskal-Wallis test in which the median of each group rather than the mean is used and thus the inclusion of the three high testosterone levels would not be problematic to analysis. This test also found no statistical difference between treatment groups ($p>0.05$). To obtain statistically useful data on serum testosterone levels in mice a far greater number of animals would be required than were available for this study.

When agents with known hepatic effects are used, impaired hepatic function or an inflammatory response can result. This can lead to a change in the levels of liver enzymes and may impact the liver's ability to remove aromatized testosterone metabolites from the serum. Since some of these metabolites can reduce testosterone levels by negative feedback on the pituitary. In these studies although a wide range of testosterone levels was observed, they all fall within the normal range suggesting that the effects of PCB treatment on the livers of exposed mice do not substantially affect circulating testosterone levels.

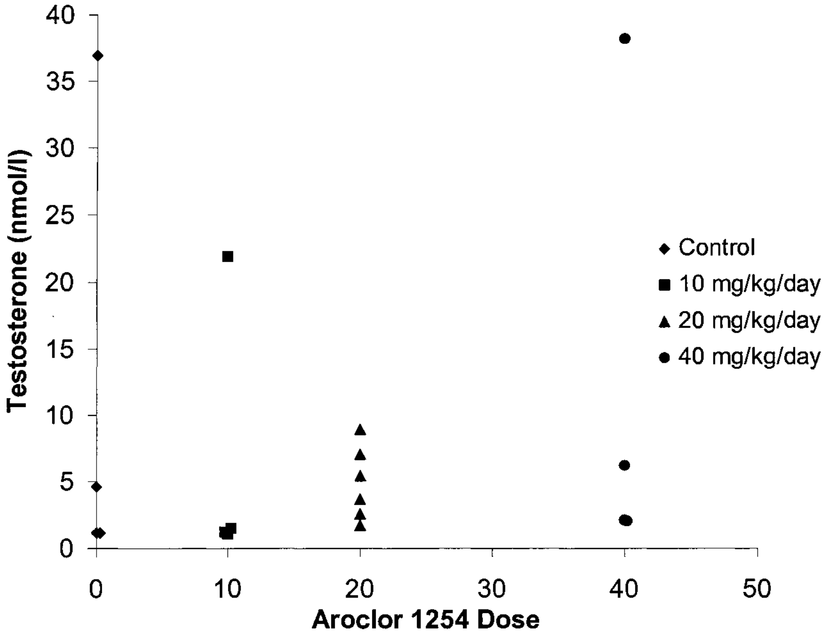


Figure 5-10: Scatter plot of serum testosterone levels from eight-week-old LPB-CAT mice.

Individuals in the dose response study treated with vehicle only (control), or 10, 20, 40 mg/kg/day Aroclor 1254.

Pathological Analysis of Histological Sections

The histological assessment of tissue samples on slides was performed by UBC veterinary pathologist Dr. Stefan Lair

Liver

Histological analysis of livers of mice from each treatment group in the dose response study was performed to confirm that the alteration in liver weight was not a result of severe toxicological changes that might affect liver function. The livers of Aroclor 1254 treated mice showed changes in the distribution of hepatocellular vacuolization. Control and 10 mg/kg/day treated mice showed no vacuolization of hepatocytes around the centrilobular veins, whereas 9% of the medium dose mice and 80% of the high dose mice livers had centrilobular vacuolization (Figure 5-11A). Control mice had diffuse or no obvious regional distribution of vacuoles in 89% (8 of 9) of the cases examined. Low dose mice had 40% (2 of 5), medium dose mice had 9% (1 of 11), and high dose mice had no cases in which there was diffuse vacuole distribution. There was also vacuolization around the periportal areas (Figure 5-11B). This was seen in 11% (1 of 9) of the control mice livers, 60% (3 of 5) in the low dose, 82% (9 of 11) in the medium dose, but only 20% (1 of 5) in the high dose. There was also an increase of infiltration of lymphocytes and small macrophages in the periportal spaces of the livers of treated mice. This was observed in 33% (3 of 9) of control mice, 20% (1 of 5) of low dose mice, 72% (8 of 11) of medium dose mice, and 100% (5of 5) of high dose mice (Figure 5-11C). Although this type of change is often considered incidental in mice, the change was more common and more severe (based on pathologist's grading) in the medium and high dose groups, which suggests an association with Aroclor 1254 treatment. This finding is consistent with that in the literature [473, 496, 499, 500]. The cellular changes were mild and were not considered clinically significant (causing disease) by the pathologist.

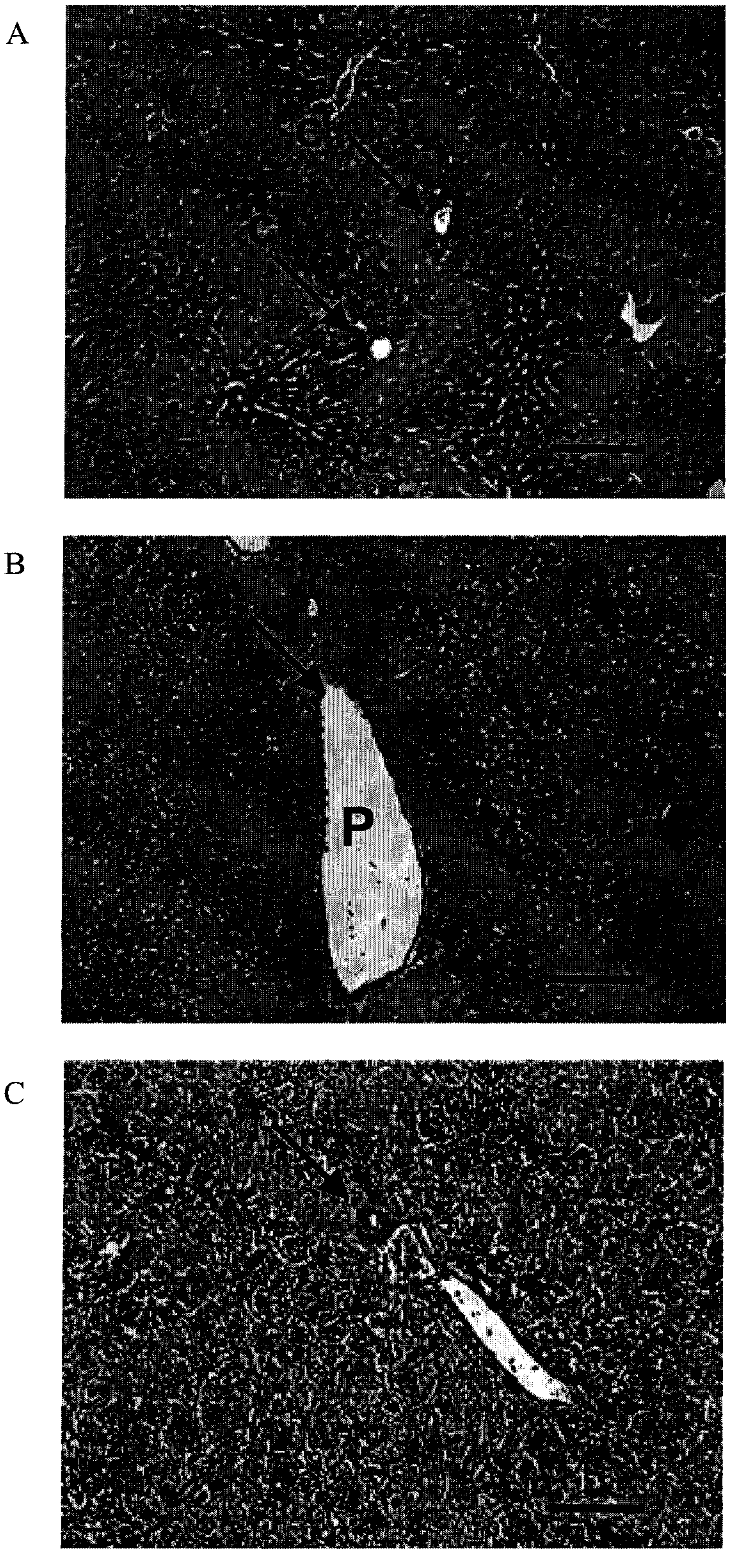

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Figure 5-11: Histological changes in livers of mice treated with Aroclor 1254.
[556] Centrilobular vacuolization in the liver of an eight-week-old mouse treated with 40 mg/kg/day of Aroclor 1254. The hepatocytes surrounding the centrilobular veins (C) are highly vacuolated (arrows) compared to cells in other regions of the liver. This vacuolization is associated with increased intracytoplasmic glycogen accumulation. This histological finding was only present in mice dosed with 20 and 40 mg/kg/day. H & E staining. Bar = 10 μM.

(B) Periportal vacuolization in the liver of an eight-week-old mouse treated with 20 mg/kg/day of Aroclor 1254. There is a high degree of vacuolization in the liver cells surrounding the periportal vein (P) relative to other areas of the liver (arrow). This type of vacuolization was observed in all treatment groups, and was most common in the liver of mice treated with 10 and 20 mg/kg/day. H & E staining. Bar = 10 μM.

(C) Accumulation of lymphocytes and small macrophages in periportal spaces (arrow) in the liver of an eight-week-old mouse treated with 20 mg/kg/day. The lesions that were observed were mild, although they were more common and more severe in the mice treated with 20 and 40 mg/kg/day. H & E staining. Bar = 10 μM.

Prostate

Histologically, there was a change in the predominant structure present in the prostates of treated mice in the dose response study. As the dose increased the percentage of cases in which dilated acini was the predominant structure also increased. Dilated acini, which were considered acini that were filled with proteinaceous material, were lined with a single row of cuboidal epithelial cells with some intraluminal infolding. A single layer of myoepithelial cells surrounded these acini. Dilated acini (Figure 5-12A) was the predominant structure in 44% (4 of 9) of control prostates, 60% (3 of 5) of low dose, 73% (8 of 11) of medium dose, and 80% (4 of 5) of high dose. In addition, there was a dose-related decrease in the percentage of cases in which non-dilated acini was the predominant structure. Non-dilated acini (Figure 5-12B) were smaller and contained less proteinaceous material than the dilated acini. They were lined by one to several rows of densely packed, low columnar to columnar, frequently disorganized, epithelial cells. There was a greater degree of infolding than in the dilated acini. One to three layers of myoepithelial cells surrounded the non-dilated acini. This structure was predominant in 56% (5 of 9) of controls, 40% (2 of 5) of low dose, 27% (3 of 11) of medium dose, and 0% (0 of 5) of high dose prostatic tissue examined.

Histological analysis of the prostates also showed intravascular multifocal lymphocytic infiltration in the prostatic stroma of mice treated with Aroclor 1254 (Figure 5-12C), while no such infiltration was seen in the prostates of control mice. This infiltration consists of random and multifocal infiltration of the interstitial tissue by discrete aggregates of small lymphocytes with undistinguishable cytoplasm. These aggregates were often in the lumen of small vessels. There was no obvious change in periductular focal lymphocytic infiltration in the prostatic stroma of the different groups. This was characterized by the presence of well-differentiated lymphocytic aggregates with scarce but distinguishable cytoplasm in the myoepithelial layers of the prostatic ductules. None of the lymphocytic infiltrates were associated with any detectable histological alterations of the glandular component of the prostate. Despite the lack of structural changes, the cause of the presence of the infiltrates has yet to be demonstrated.

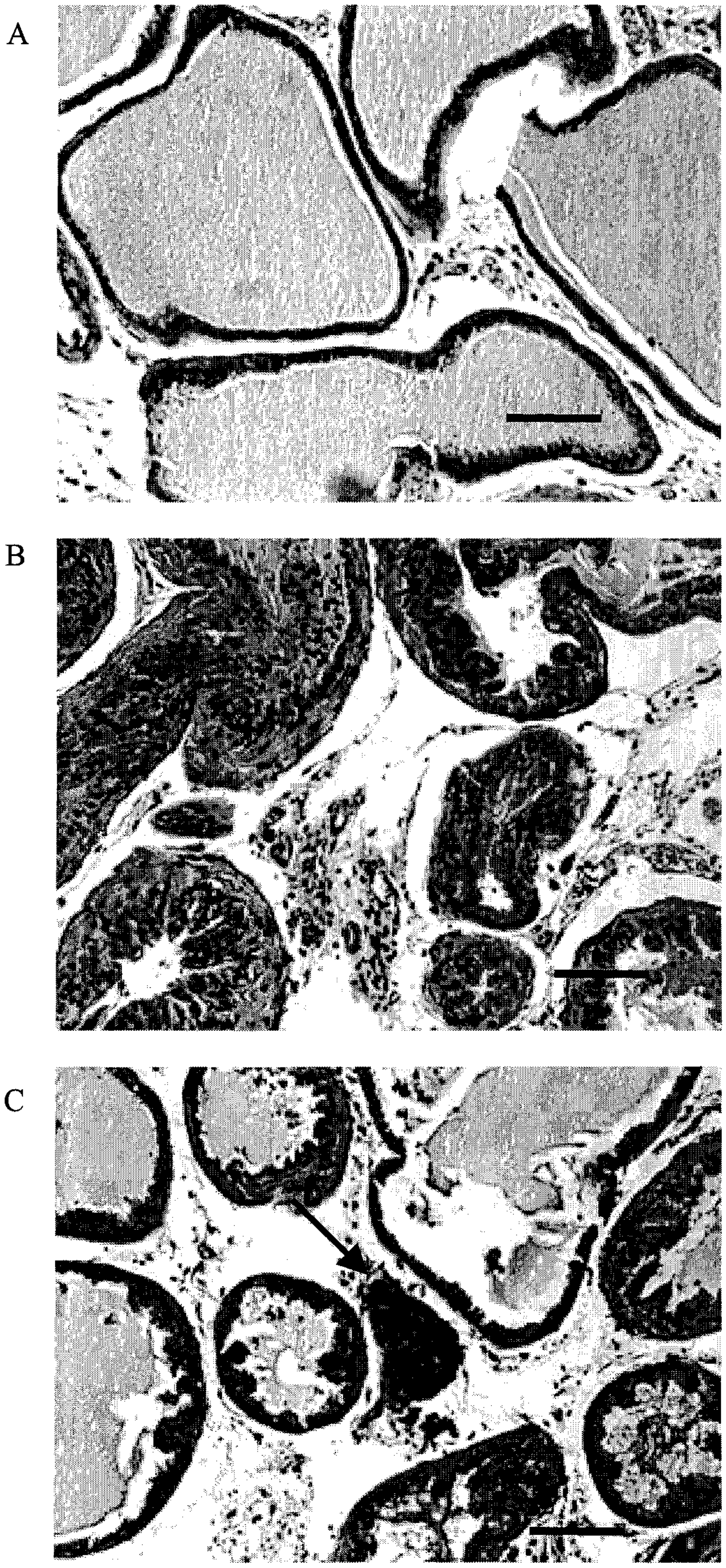
A
B
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Figure 5-12: Histological changes in the prostates of mice treated with Aroclor 1254. [556] Dilated acini of the prostate of an eight-week-old mouse control group mouse. This was found to be the predominant structure in the mouse prostates in an Aroclor 1254 dose related manner. H & E staining. Bar = 5 μM.

(B) Non-dilated acini of the prostate of an eight-week-old control group mouse. This was found to be the predominant structure in the mouse prostates in an inverse Aroclor 1254 dose related manner. H & E staining. Bar = 5 μM.

(C) Infiltration of discrete aggregates of small lymphocytes (arrow) in the interstitial tissue of the prostate of an eight-week-old mouse treated with 40 mg/kg/day of Aroclor 1254. These infiltrates were present in the prostates of treatment mice but were not detected in the prostates of control mice. H & E staining. Bar = 5 μM.

Testes

Testes of mice from the different dose groups were also examined and found to be histologically normal is all cases. The tissue consisted of numerous well-differentiated seminiferous tubules separated by the septula testis. The stroma contained a normal number of Leydig cells. Active spermatogenesis was found in all tubules, as characterized by the presence of numerous spermatogenic cells.

5.3 PCB Congeners

The results presented here indicate that Aroclor 1254 is a weak agonist of AR mediated gene transactivation. The various competitive binding experiments undertaken indicate that several Aroclor 1254 components are able to effectively compete with the natural ligand for its AR binding site and act as an agonist in the absence of androgen but antagonist the action of physiological androgens when thay are added. Since Aroclor 1254 produced an agonist type response at high concentrations, it can be labelled as a partial agonist. However due to the complexity of the Aroclor 1254 mixture and the diverse array of congeners it comprises it is possible that there are multiple active components, with different activities. Some of the congeners may act as partial or weak agonists while others are pure antagonists. Therefore, it may be more accurate to state that the sum effect of the PCB congeners in Aroclor 1254 is that of a weak agonist.

In PC-3 cells Aroclor 1260 had no clear effect upon AR mediated firefly luciferase expression. However in LNCaP experiments performed by Cheryl Portigal Aroclor 1260 exhibited antagonistic effects in the presence of DHT, albeit weaker then Aroclor 1254 [200]. In HeLa cell competitive binding experiments Aroclor 1260 was also an inferior competitor for DHT when compared to Aroclor 1254, suggesting that it has a lower content of congeners with an affinity for AR. To understand the differences between the two Aroclor mixtures a comparison of the composition of the two Aroclor mixtures (1254 vs. 1260) was undertaken to identify congeners that occurred at significantly different concentrations. Of greatest interest were those congeners detected in Aroclor 1254 at several fold higher levels than in Aroclor 1260, since these would be good candidates for AR ligands. Table 5-4 is a summary of those congeners present in the Aroclor mixtures at different levels [236]. For example PCB 31 comprises 0.7% of Aroclor 1254, yet is not detected in Aroclor 1260. PCB 42 comprises 2.2% of Aroclor 1254, as compared with 0.7% of Aroclor 1260. Thus, the congeners composition defines each Aroclor mixture and these variations in composition underlie the differences in activity between Aroclors.

Major PCB congener differences between Aroclor mixtures 1254 and 1260			
Congener IUPAC #	PCB Structure	Aroclor 1254 mol %	Aroclor 1260 mol %
31	2,4',5-trichlorobiphenyl	0.72	Not detected
42	2,2',3,4'-tetrachlorobiphenyl	2.18	0.66
49	2,2',4,5'-tetraCB	1.63	0.44
66	2,3',4,4'-tetrachlorobiphenyl	2.24	0.22
70	2,3',4',5-tetrachlorobiphenyl	4.75	0.85
72	2,3',5,5'-tetrachlorobiphenyl	1.01	0.28
85	2,2',3,4,4'-pentachlorobiphenyl	2.15	0.31
87	2,2',3,4,5'-pentachlorobiphenyl	3.81	1.10
97	2,2',3',4,5-pentachlorobiphenyl	2.59	0.63
99	2,2',3',4',5-pentachlorobiphenyl	6.10	0.82
118	2,3',4,4',5-pentachlorobiphenyl	8.09	2.00
121	2,3',4,5',6-pentachlorobiphenyl	3.51	0.57
128	2,2',3,3',4,4'-hexachlorobiphenyl	1.31	0.47
138	2,2',3,4,4',5'-hexachlorobiphenyl	4.17	5.01
168	2,3',4,4',5',6-hexachlorobiphenyl	4.23	0.59
193	2,3,3',4',5,5',6-heptachlorobiphenyl	2.30	Not detected
198	2,2',3,3',4,5,5',6-octachlorobiphenyl	1.00	0.15

Table 5-4: Summary of PCB some congener differences between Aroclors 1254 and 1260. Congeners shown generally comprise more than 1% by molarity of Aroclor 1254 and are three or more times less prevalent in Aroclor 1260. PCB 31 was included even though it is present at less than 1% since it is the most highly represented trichlorobiphenyl congener in Aroclor 1254.

5.3.1 Congener results

Initially three PCB congeners from Table 5-4 were selected and tested in two cell lines (PC-3 and LNCaP) for their effect upon AR mediated transcription of the firefly luciferase gene. The congeners chosen were PCB 31, (2,5,4'-trichlorobiphenyl), PCB 42, (2,2',3,4'-tetrachlorobiphenyl), and PCB 99, (2,2',4,4',5-pentachlorobiphenyl).

In both PC-3 and LNCaP cells, both PCB 31 and 42 produced significant antagonistic effects versus 1 nM DHT. Again, LNCaP cells were more sensitive with effects seen at concentrations above 100 nM and 50% inhibition produced at 700 nM by PCB 42 and 8μM by PCB 31 (Figure 5-13). In PC-3 cells the congeners exhibited less antagonism although for both PCB 31 and 42 a statistically significant reduction in DHT induced firefly luciferase activity was measured when a 100 nM concentration or higher was applied. PCB 99 did not produce any statistically significant response in either cell line. In experiments without DHT, none of the congeners produced significant induction of firefly luciferase at concentrations tested, up to 100 μM (results not shown).

Five additional congeners from Table 5-4 were tested in LNCaP cells for their impact on AR mediated transactivation (Figure 5-14). The congeners used were PCB 118, (2,3',4,4',5-pentachlorobiphenyl), PCB 128, (2,2',3,3',4,4'-hexachlorobiphenyl), PCB 138, (2,2',3,4,4',5'-hexachlorobiphenyl), PCB 168, (2,3',4,4',5',6-hexachlorobiphenyl), and PCB 198, (2,2',3,3',4,5,5',6-heptachlorobiphenyl). All congeners except for PCB 198 exhibited antagonist activity versus 0.05 nM DHT in the order (when tested at 10 μM) PCB 168> PCB 128 > PCB 138 > PCB 118 > PCB 198. PCB 168 reduced DHT induced luciferase activity by 70% when added at 10 μM compared to PCB 118, which at the same concentration reduced luciferase by 45%. PCB 198 had no effect upon luciferase activity in the presence of DHT and none of the congeners was found to affect luciferase activity when added to the cells alone in the absence of DHT (results not shown).

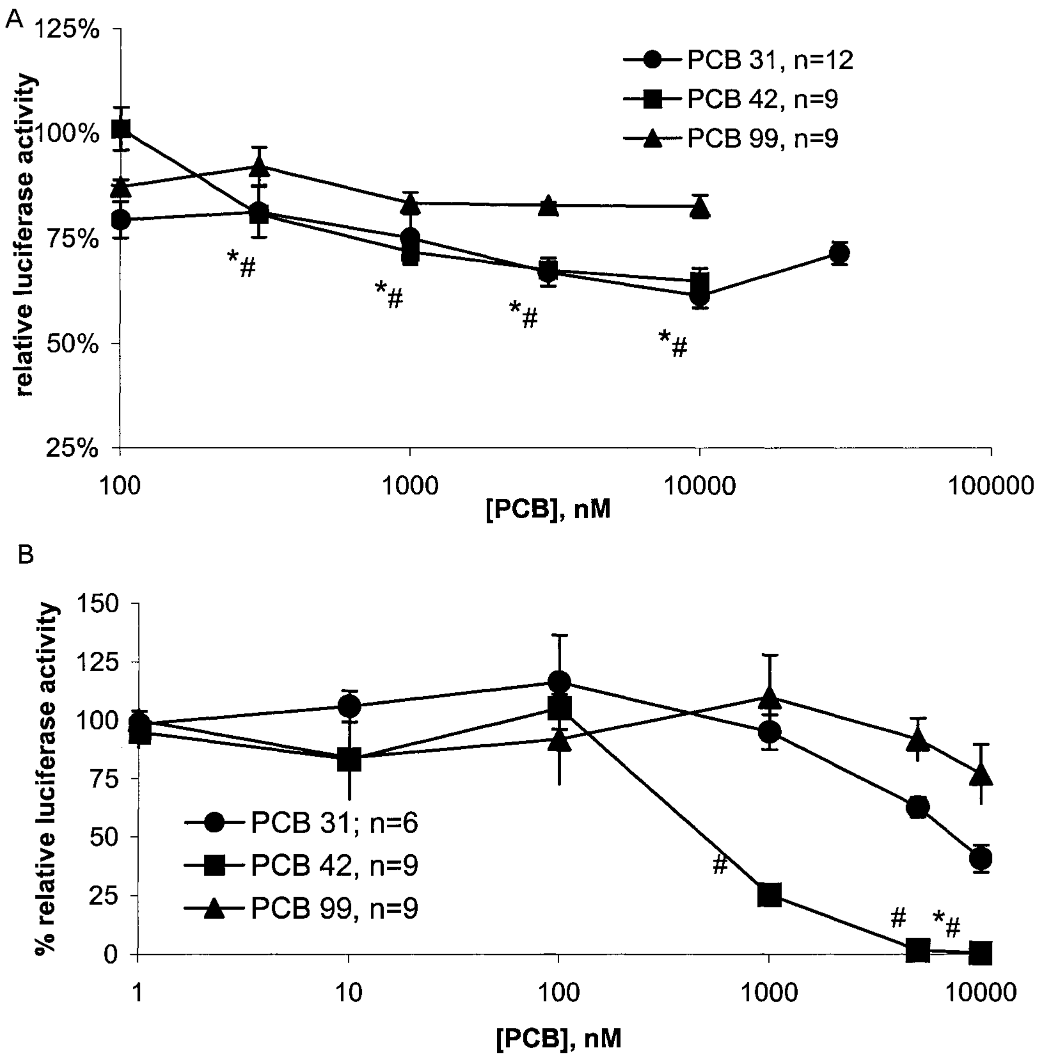

Figure 5-13: The effects of three PCB congeners on androgen driven gene expression. In AR transfected PC-3 cells with1 nM DHT added (A), and in AR transfected LNCaP cells with 0.05nM DHT added (B). Data points at which PCB 42 (#) and PCB 31(*) differ significantly ($p<0.05$, using Student's T-test) from the control mean (no PCB added, set at 100% relative luciferase activity) are indicated. Number of individual wells (n=), from multiple experiments, used to derive the graph is shown.
Error bars indicate the standard error of the mean (SEM).

PCB congeners 31 and 42 were subjected to the same set of binding experiments as the Aroclors. In the first method using AR transfected PC-3 cells it was found that both congeners were able to displace ^{3}H-DHT from the PC-3 cells in a dose dependent manner (Figure 5-15A). To further investigate the role of AR in this displacement, HeLa cells stably transfected with AR were treated with tritiated DHT and PCBs 31, 42 and 99 were added as cold competitors. In this case only PCB 42 measurably reduced the amount of DHT bound by the HeLa cells (Figure 5-15B). PCB 31 seemed to cause an increase in the cell bound radiolabel, although this was not statistically significant while PCB 99 produced no effect.

Finally, to determine if there was a specific direct interaction with the AR ligand-binding domain (AR-LBD) purified recombinant protein (PanVera) was mixed with radiolabeled ligand and PCBs 31, 42 and 99 were added (Figure 5-15C). Again only PCB 42 was found to bind the AR-LBD and displace the radiolabeled ligand while both PCBs 31 and 99 produced no effect. The AR-LBD experiment was repeated for the other five congeners listed above and in this case only PCBs 128 and 138 produced a significant decrease in the binding of radiolabeled ligand. The other three congeners produced no substantial effect (Figure 5-16).

The three congeners, PCBs 31, 42 and 99 were also investigated for their interactions with GR. All three were added to GR transfected PC-3 cells to study their effect upon GR mediated gene transcriptional activation. None of the congeners produced a statistically significant effect (Figure 5-17A), although PCB 42 did seem to generate some antagonism at the highest concentration tested (30 μM).

When the activity of PCBs 31 and 42 on GR was further investigated in LNCaP cells it was found that PCB 42 acted as an effective agonist but only in an additive manner in the presence of DEX. PCB 31 produced no effect either with or without the addition of DEX (Figure 5-17B and results not shown). When tested in the PC-3 ligand displacement assay both congeners 31 and 42 were able to reduce the amount of 3H-DEX associated with GR transfected cells by more than 50% (Figure 5-17C).

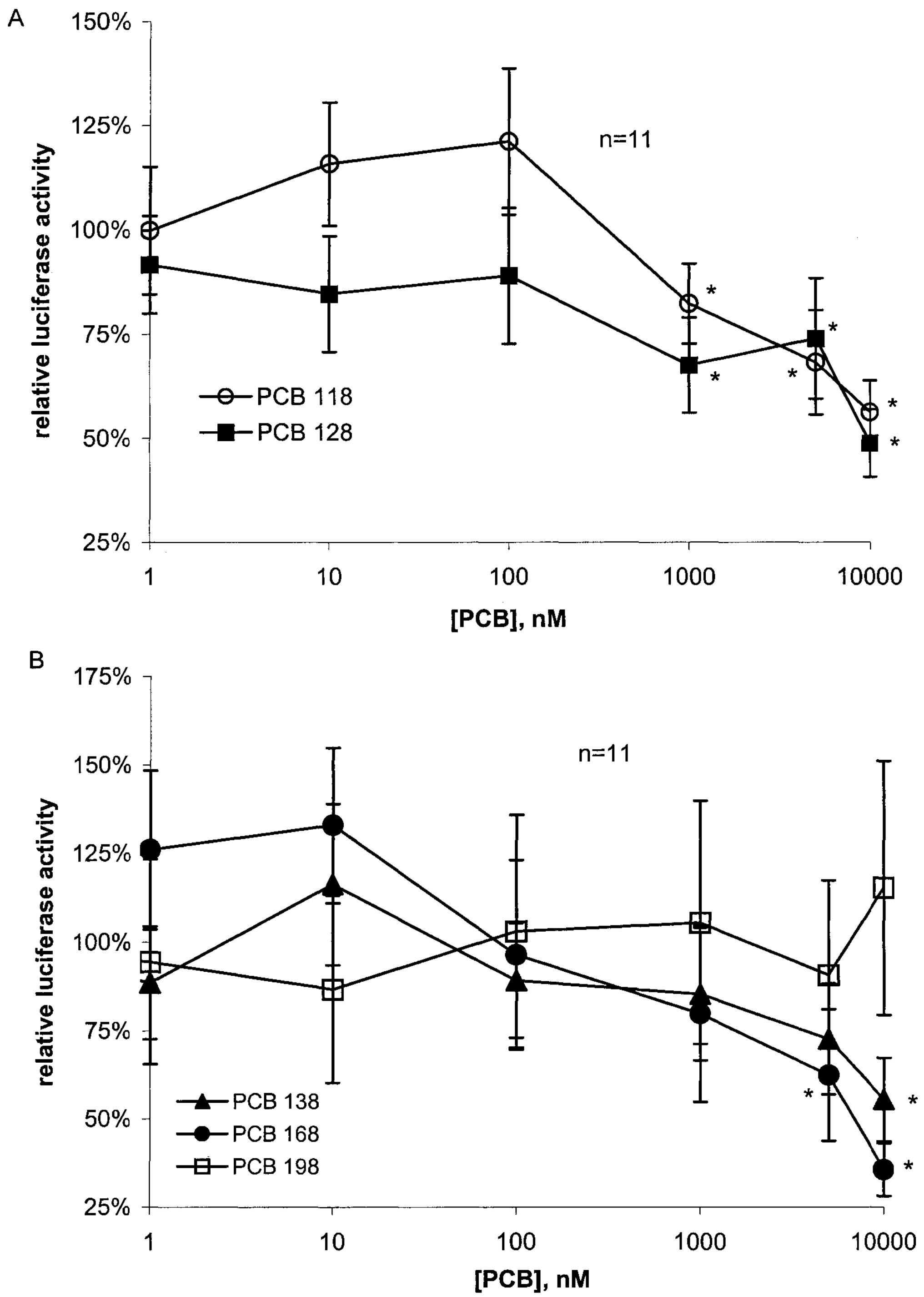

Figure 5-14: The effects of five PCB congeners on androgen driven luciferase expression in LNCaP cells with 0.05nM DHT added. The * indicates a value is significantly different ($p<0.05$, using Student's T-test) from control (no PCB added, set at 100% relative luciferase activity). Number of individual wells (n=), from multiple experiments, used to derive the graph is shown. Error bars indicate the standard error of the mean (SEM).

N.B. at no point does PCB 198 differ significantly from the control

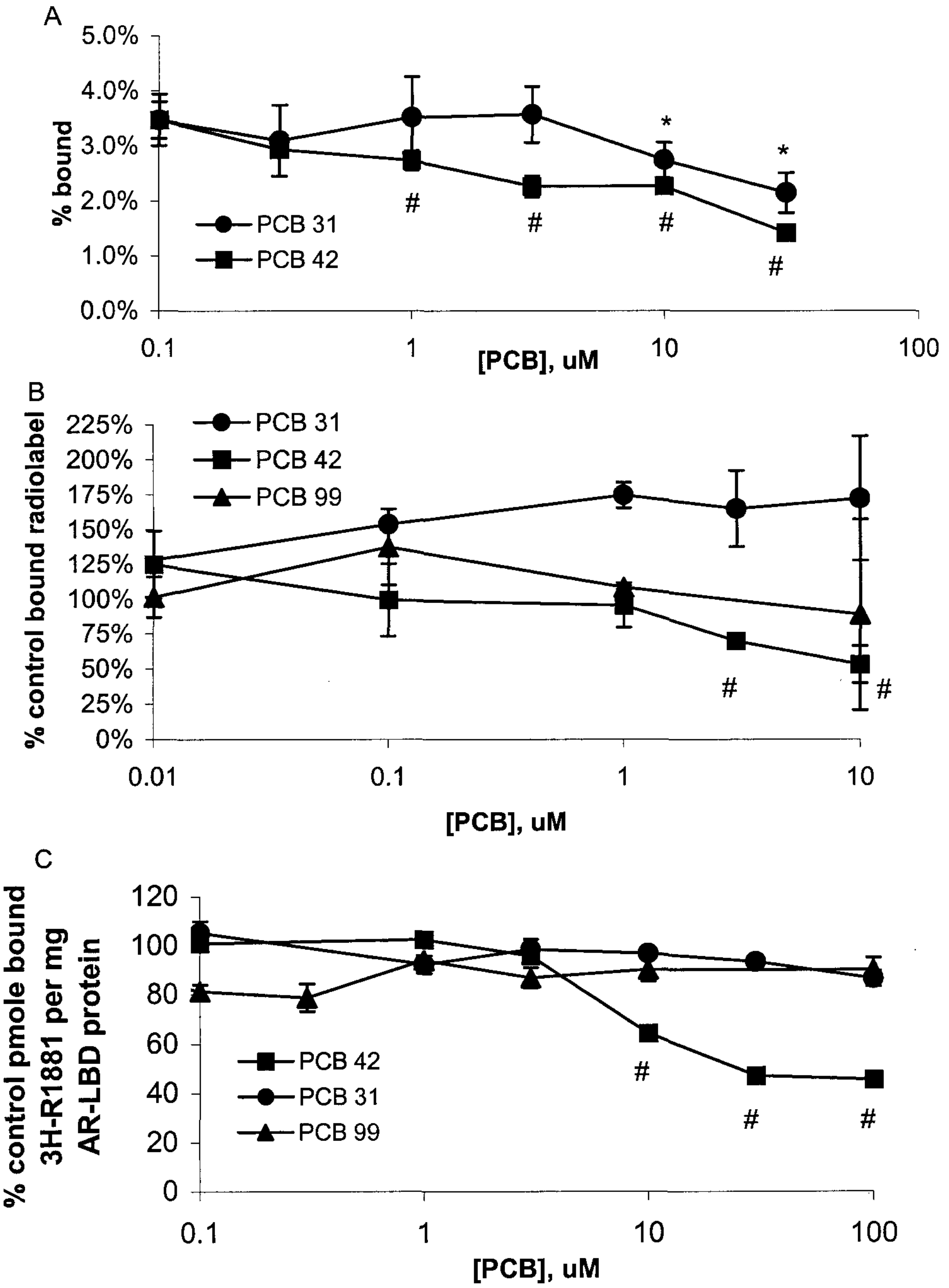

Figure 5-15: The ability of PCB congeners, 31, 42 and 99 to interfere with the binding of DHT in transiently AR-transfected PC-3 cells (A), stably AR-transfected HeLa cells (B), and to purified recombinant AR-LBD.Values which differ significantly ($p<0.05$, using Student's T-test) from the control (no PCB added) are indicated by * (PCB 31) and # (PCB 42). Error bars indicate the standard error of the mean (SEM).

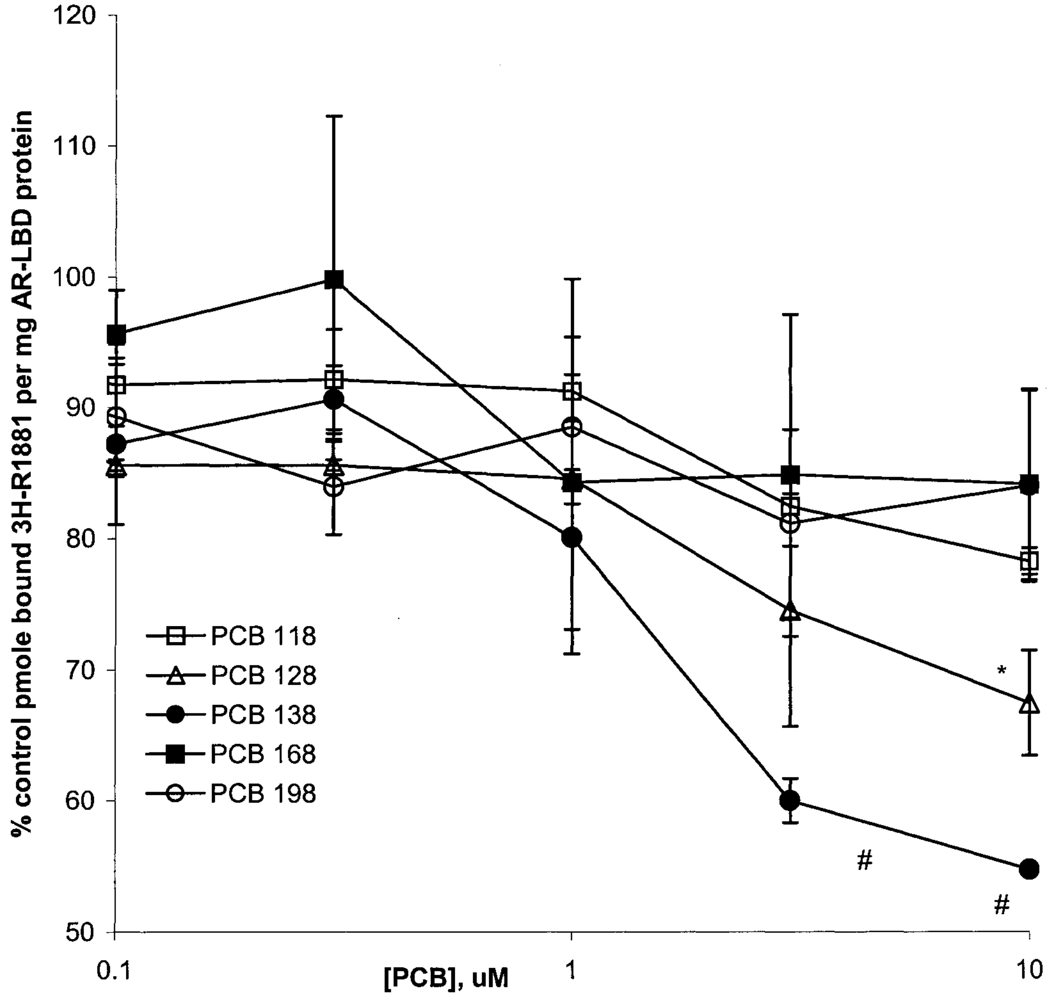

Figure 5-16: The ability of PCB congeners to compete with R1881 for binding to purified recombinant AR-LBD. Values which differ significantly ($p<0.05$, using Student's T-test) from the control (no PCB added; set at 100%)are indicated by * (PCB 128) and # (PCB 138). Error bars indicate the standard error of the mean.

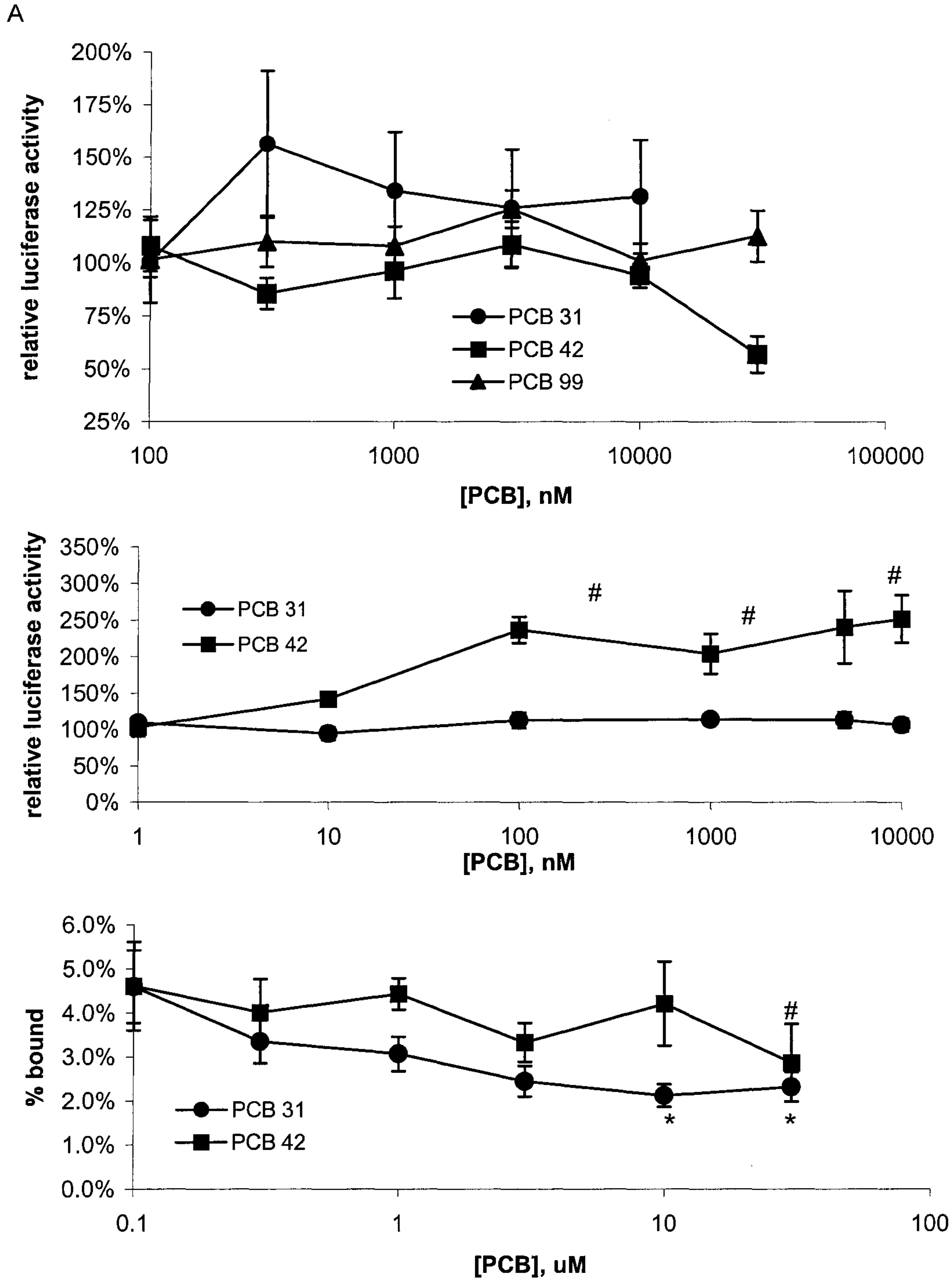

Figure 5-17: The effects of PCB congeners 31,42, and 99 on GR. PC-3 cells transfected with GR and exposed to varying concentrations of PCB 31, 42 and 99 in the presence of 1nM DEX,(A); LNCaP cells transfected with GR and exposed to PCB 42 and 1nM DEX, (B); PC-3 cells transfected with GR and exposed to PCBs 31 and 42 with 3H-DEX (C).

Values which differ significantly ($p<0.05$, using Student's T-test) from the control (no PCB added) are indicated by * (PCB 31) and # (PCB 42). Error bars indicate the standard error of the mean.

5.3.2 PCB 42 *in vivo*

The above *in vitro* studies provide evidence that certain individual congeners are able to bind to, interact with and modulate the activity of steroid hormone receptors. It was decided therefore to examine the effects of one of the congeners *in vivo* in a similar manner as had been done for Aroclor 1254. For this study PCB 42 (2,3,2',4'-tetrachlorobiphenyl) was selected since it seemed the most potent AR ligand from those tested. An expanded selection of organs were weighed, including brain, lung, thymus, heart and adrenal in addition to liver, testes, epididymis and prostate. Mice were dosed with oil vehicle only, or PCB 42 in oil at 2.5, 5.0 and 10.0 mg/kg/day. Dams were treated from day of pairing until the pups were weaned from which point the offspring males were given the corresponding dose until sacrifice. Mice from all dose levels were sacrificed at 4 or 8 weeks of age, control and low dose mice were also sacrificed at 12 weeks of age with 5 to 7 mice for each dose at each age.

Body weight data was collected throughout for mothers and pups. Although there are some reports in the literature of PCB exposure causing a decrease in body weight, PCB 42 had no effect upon the body weights of the mice during the study. Since the size of many organs is in proportion to an individual's body weight, the organ weights reported here were adjusted for body weight before analysis.

Liver

Liver weight was found to increase slightly with PCB 42 exposure although this was only significant in the 10 mg/kg/day dose for 4-week-old mice, and in the 5 gm/kg/day dose for the 8-week-old mice (Table 5-5). In 12-week-old mice, the liver weight for the 2.5 mg/kg/day was actually decreased relative to the controls. Since this liver enlargement was marginal and did not correspond to any apparent histological changes in the liver it is unlikely have any adverse affects upon the mice, or impact upon the experimental outcomes.

Kidney

Kidney weight increased significantly in mice exposed to either the 2.5 mg/kg/day and 10 mg/kg/day doses of PCB 42 at 4 weeks of age and persisted in those given the high dose at 8 weeks (Table 5-5).

Mice at four weeks of age

Organ	CONTROL (N=7)	2.5 mg/kg/day (N=7)	5.0 mg/kg/day (N=5)	10 mg/kg/day (N=7)
Prostate	0.270±0.022	0.412±0.104	0.274±0.019	0.375±0.090
Epididymis	0.565±0.031	0.681±0.040*	0.539±0.014	0.675±0.087
Testis	2.271±0.224	2.948±0.160*	2.444±0.089	3.088±0.093*
Kidney	6.138±0.093	6.734±0.206*	6.238±0.132	7.088±0.145*
Liver	52.64±0.84	50.00±2.75	51.02±2.07	57.82±1.12*

Mice at eight weeks of age

Organ	CONTROL (N=7)	2.5 mg/kg/day (N=7)	5.0 mg/kg/day (N=6)	10 mg/kg/day (N=7)
Prostate	0.433±0.022	0.475±0.027	0.558±0.050	0.490±0.043
Epididymis	2.294±0.665	4.237±0.277*	3.956±0.635	4.062±0.281*
Testis	3.890±0.167	3.999±0.286	3.973±0.102	4.355±0.070*
Kidney	7.372±0.263	7.483±0.203	7.193±0.223	8.032±0.182*
Liver	46.53±1.94	48.26±0.57*	52.09±0.98*	51.15±1.49

Mice at twelve weeks of age

Organ	CONTROL (N=6)	2.5 mg/kg/day (N=7)
Prostate	0.507±0.033	0.480±0.036
Epididymis	0.833±0.054	1.087±0.053*
Testis	3.072±.0322	4.209±0.158*
Kidney	6.974±0.309	7.442±0.223
Liver	46.96±1.10	42.36±1.03*

Table 5-5: Adjusted organ weights from LPB-CAT mice treated with PCB 42. Corrected (organ weight/body weight in grams/gram body weight x10^4) for mice treated for eight weeks of age with canola oil or PCB 42 at 2.5, 5.0, 10 mg/kg/day. Values are mean ± SEM. *$p<0.05$

Testes

At all three ages it was found that the weights of the testes from PCB 42 treated mice had increased (Table 5-5). In the 4-week-old mice, this increase was statistically significant in the 2.5 and 10 mg/kg/day treatment groups (p=0.0316 and p=0.0097 respectively) while in the 8-week-old mice the increase was significant in the 10 mg/kg/day group (p=0.0332). In the 12-week-old mice, the 2.5 mg/kg/day mice, the only treatment group, also had significantly enlarged testes (p=0.0157).

Epididymides

As with the testes, the weights of epididymides at all three-time points were found to increase (Table 5-5). In the 4 week old mice the increase was statistically significant in only the 2.5 mg/kg/day group (p=0.0408), while in the 8 week old mice those treated at both the 2.5 and 10 mg/kg/day showed a statistically significant weight increase (p=0.0272 and p=0.0401 respectively). Although the increase in weight was small in the 12-week-old mice, the treatment group still had significantly larger epididymides (p=0.0064).

The effect of PCB exposure on the epididymides seems to be multifaceted. In addition to the increased weight, the pathologist's examination revealed changes in spermatogenesis. Overall spermatogenesis decreased with PCB 42 treatment although there may be an apparently contrary tendency towards precocious spermatogenesis at the highest exposure level, in the 10 mg/kg/day mice, since one individual from this group was found to have commenced spermatogenesis at 4 weeks of age, a phenomenon not normally seen in such young mice and not found in any other individual in this study. Clearly, a single individual does not make a statistically significant result but this finding is intriguing and may warrant further investigation.

Prostates

Neither changes in prostate weight nor significant changes in prostate histology were found in mice exposed to PCB 42. Correspondingly, no significant change in CAT expression in the prostate tissue resulted from PCB exposure. PCB congener 42 is an excellent candidate for Eukaryotic metabolism having several sites for oxidative attack by

P450 enzymes. It is likely therefore that the congener was largely eliminated soon after ingestion by the mice.

In common with the findings in Aroclor 1254 and nonylphenol treated mice as well as hexachlorobenzene treated mice (Jody Saito), intravascular multifocal lymphocyte infiltration was found in several of the treated mice. This phenomenon was exhibited in two 10 mg/kg/day and one 2.5 mg/kg/day dosed mice from the four-week-old treatment group and two 10 mg/kg/day and one 5 mg/kg/day dose mice from the eight week old treatment group as well as three mice in the 12 week old treatment group. In contrast, this type of lymphocyte infiltration was not observed in any of the control mice.

5.4 Discussion

Although considerable evidence has accumulated indicating that PCBs can interfere with estrogen, thyroid and glucocorticoid mediated gene transcription, little work has been published on the effects of PCBs directly on the androgen axis. The results shown here clearly indicate that some PCBs are able to interact directly and specifically with the androgen receptor and disrupt the function of androgens both *in vitro* and *in vivo*.

All the Aroclor mixtures tested (1242, 1248, 1254 and 1260) contained active components able to interfere with DHT at the level of the AR. Further analysis of the role of individual congeners in this activity indicates that despite the promiscuity of the AR, there is clearly a requirement for specific structural motifs to achieve a stable ligand-receptor complex. Non-ortho co-planar PCBs appear to be poor ligands for the AR, whereas those with a PCB 42 (2,2',3,4'-tetrachlorobiphenyl) core appear to form the best ligands. Nevertheless, both congeners 118 and 168, which deviate from this core pattern, appear nevertheless to be active in transcriptional activation assays. Both are comparably able to inhibit DHT mediated firefly luciferase gene expression as PCB 42, 128 and 138, but do not apparently bind well to the AR-LBD since they do not effectively compete with radiolabeled ligand for binding. Perhaps these two congeners bind to the AR at another site (not present in the truncated recombinant protein used in the assay) or function via another mechanism.

Within any Aroclor mixture there is a diverse array of congeners represented which may manifest equally diverse effects upon a steroid receptor. It is likely that many have little to no effect upon a given receptor, others may be pure antagonists, and some may conceivably be partial or weak agonists which in isolation and at a high enough concentration may be capable of inducing gene transactivation through the receptor.

PCBs have remained a public concern, as they are stable and persistent environmental contaminants that due to their lipophilicity migrate to fatty tissues and bioaccumulate in the food chain. There is a highly varying range of PCB levels reported in serum and human breast milk, largely due to differences in diet and environmental and occupational exposure. The National Human Adipose Tissue Survey found levels ranging as high as 1700 ppm, which is approximately equivalent to the higher levels of PCBs used in this study [236]. The prostate is a highly lipophilic gland, however specific measurement of levels of PCB congeners have not been thoroughly documented. The range of PCB levels tested in this study have been linked to the ability of PCBs to interfere with endocrine homeostasis. The role of PCBs and PCB metabolites as estrogens has been previously documented [402], however, the effects of PCBs on androgen mechanisms has not been thoroughly investigated. Bioassays using reporter genes and AR ligand displacement were used in this study to gain new information pertaining to the capacity of PCBs to influence steroid hormone action through the androgen axis *in vitro*. The evidence presented here demonstrates an ability of PCBs to alter androgen-regulated processes *in vitro* at the molecular level and *in vivo*. The transactivation data demonstrates that some PCBs can act as weak AR agonists in the absence of DHT, and are antagonistic to AR activity in the presence of DHT. Interestingly the effects we have observed were almost exclusively in AR assays, suggesting PCBs interact poorly with GR.

All the Aroclor mixtures tested produced an antagonistic effect in the presence of DHT when cells were transfected with AR, although the specifics of the trend varied between mixtures. The results from our competition studies indicate that Aroclor 1242, Aroclor 1248, Aroclor 1254, and Aroclor 1260 competitively inhibit DHT from binding to AR. Together these results suggest that one or more specific congeners found in the Aroclor mixtures possess androgenic activity rather than representing a general property of all PCBs. Previously, some hydroxylated PCB metabolites have been shown to bind to ARs in the kelp bass and Atlantic Croaker causing 50% displacement of ^{3}H-testosterone [369]. The ligand displacement assay results here show that specific PCB congeners with the core of chlorine substitutions at 2, 2', 3 and 4' can reduce natural ligand binding to the mammalian AR. These results, combined with the ability of the Aroclors to alter the androgen driven luciferase reporter gene suggest that PCBs can affect AR activity in a wide range of species. As PCBs degrade extremely slowly, and highly chlorinated congeners remain in tissues for months or years before they are metabolized, an accumulation of these chemicals to levels where androgenic activities have detectable and detrimental effects seems probable.

These results demonstrate that some PCBs may act as weak AR agonists in the absence of DHT, and may antagonize AR activity in the presence of DHT, the implications of which are profound during development. The action of PCBs as weak androgens may have implications in humans and wildlife that are exposed to these compounds throughout their development. Androgen sensitive organs such as the prostate may undergo altered development *in utero* when there are low levels of androgens present, as well as during puberty when there is a surge of circulating androgens in serum that initiate prostate growth and development. There may also be an effect of such antiandrogens in later life, as an alteration in normal prostate development at earlier stages may have implications on changes in prostate growth in later life [560]. Broad based studies performed in rats exposed to Aroclor 1254 with gross morphological endpoints found that 1254 exposure resulted in decreased prostate weight, however this was not mechanistically investigated [546]. Similarly, a PCB mixture similar to 1242 administered to rats resulted in decreased weight of androgen dependent male reproductive organs. Administration of PCB 126 in rats also decreased prostate weight, however this effect may have been mediated by AhR receptor [561].

Clearly, if particular PCB congeners can bind to AR and inhibit its activity, it will be important to extend our findings into specific AR-regulated functions *in vivo* to ascertain if PCBs can directly affect AR-driven processes *in vivo*. Since a number of PCB congeners are also known to activate AhR [562-564] it is plausible that AR may be antagonized indirectly by PCBs that serve as AhR ligands. Activation of AhR is known to interfere with the activity of AR and ER. Therefore, complex mixtures of PCBs that contain both AhR-ligands and AR or ER ligands may show suppression of transcriptional activity via multiple mechanisms.

Some congeners have been shown to alter AR protein levels in the prostate due to developmental exposure and other congeners have been shown to increase the metabolism of androgens *in vivo*. Therefore, the net result *in vivo* of the mechanisms involved in the action of mixtures will be difficult to interpret.

This study showed marked changes in mouse prostates in response to Aroclor 1254 treatment. A significant decrease in ventral prostate weights and CAT activity was detected in mice treated to eight weeks of age at a dose of 10 mg/kg/day. This is a strong indication that Aroclor 1254 has antiandrogenic activity in the adult murine system *in vivo*, and is consistent with the data showing Aroclor 1254 as a weak antiandrogen *in vitro*.

The decline in prostate weights in treated mice indicate that Aroclor 1254 has a major influence on reproductive development, and can influence the responsiveness of androgen target organs. Studies on androgen related organs in rodents treated with PCBs have shown considerably variable effects. Mice treated in our study showed a reduction in ventral prostate weight. Another study found that rats dosed with Aroclor 1254 at 8, 32, and 64 mg/kg/day during lactation had significantly lower prostate weights with fewer acini and flattened epithelial cells compared to controls at day 165 [546]. However, more recently female rats treated with Aroclor 1254, 50 days prior to birth until parturition produced male offspring with no significant reduction in ventral prostate weight at 170 days of age [547]. This may indicate that the developmental window during which the animal is exposed is critical to the effect produced.

The possibility of liver toxicity was an important consideration in these studies. The effects of PCB treatment could adversely affect the livers of treated mice and produce changes in hormone metabolism, which might indirectly affect hormone target organs like the prostate. The observed increase in liver weight in PCB treated mice was consistent with other studies that show an increase in relative liver weights in rats treated with various Aroclor mixtures [473, 497-500, 566]. Histological effects include hepatocyte hypertrophy, fat deposition, fibrosis, necrosis, and changes in serum levels of liver associated enzymes indicative of possible hepatocellular damage [236]. There are no previous reports of specific patterns of hepatocellular vacuolization due to PCB treatment. Although Aroclor 1254 treatment altered hepatocellular vacuolization, this change is not considered to cause adverse affects in liver function. In addition, PCB 42 produced only marginal weight changes in the livers of treated mice and no histological or physiological effects were detected. In addition serum testosterone levels are within a normal range for all dose levels and there was no significant change in the treated mice further supporting the hypothesis that the PCB effects were as direct anti-androgens on the prostate. Therefore, although mild changes were observed in some livers in this study, the results do not suggest that liver function, including the ability to metabolize testosterone, is significantly compromised by Aroclor 1254 treatment.

In the PCB 42 study mouse testis weight increased with exposure, whereas in the Aroclor 1254 study there was no change in testis weight observed in treated mice. One study in rats also found no change in testis weights in animals dosed post-weaning with Aroclor 1254 for 35 to 105 days [473]. However, other studies have found significantly increased testes weights in rats receiving doses of 32 mg/kg/day Aroclor 1254 or higher [567] and in rats treated with Aroclor 1254 from birth to day 25, and examined at day 135 [425]. A decrease in caudal epididymal weights in rats exposed to Aroclor 1254 has been reported [473], whereas this study found that epididymis weight increased with PCB 42 exposure but was not significantly changed by Aroclor 1254. These variations in organ and body weight effects are likely due to a variety of factors including age and length of dosing, and the species and strain studied.

The congener study found that exposure to PCB 42 affected spermatogenesis in the mouse epididymis. In general, spermatogenesis was slightly impaired in treated mice except that one of the 10 mg/kg/day dosed mice exhibited precocious spermatogenesis at four weeks of age. Spermatogenesis is regulated by androgens in both the testis and the epididymis [568] indicating that the antagonist effects seen for PCB 42 *in vitro* may be reproduced *in vivo*. This may have implications for the fertility of these mice; although a full reproductive study would be required to establish such an effect.

These studies have identified a unique histological finding of lymphocytic infiltrates in the prostates of treated mice. This effect of PCBs on prostate development and function warrants further investigation, to determine the cause and role of such infiltrates. The presence of these lymphocytes may be due to apoptotic events, which are known to occur in the rodent during rapid prostate regression induced by androgen withdrawal [569]. The presence of lymphocytic infiltrates may be indicative of alterations in prostatic function that could also affect sperm motility and ultimately reproductive fitness. There was also a dose-related increase in the presence of dilated acini and a decrease in non-dilated acini in the prostates of Aroclor 1254 treated mice. This seems to contrast with the corresponding decrease in prostate weights with increasing Aroclor 1254 dose. Together these results seem to suggest that the net effect of Aroclor 1254 on the prostate is to accelerate maturation of the gland thereby restricting the growth and size of the gland.

One consideration when working with complex industrial mixtures, such as Aroclors, is the difficulty of discerning which specific congeners, or combination of congeners, are responsible for the results observed. The induction of transcription as well as the prevention of AR ligand binding may be due to the action of certain congeners on their own, or a combined effect of many components of the Aroclor mixtures.

Structurally, PCB congeners can be subdivided into two broad classes on the basis of the orientation of the biphenyl rings as either co-planar or non-planar. In general, co-planar PCBs are more likely to interact with AhR and non-planar PCB congeners with chlorination in the ortho positions on the other hand may be more likely to interact with steroid receptors, as demonstrated for ER. From our results, it appears that the non-planar orientation with two ortho substitutions, combined with one substitution at the meta and para position, respectively, binds AR with high affinity. This basic substitution pattern of congener 42 also seems to be predictive of the binding affinities of higher chlorinated congeners, explained by the fact that only congeners with this basic substitution pattern seem to have any affinity for AR. This structure-activity relationship could possibly be used to establish which PCB congeners will exhibit the highest androgen modulating effects, presumably because of their strong interaction with AR.

Comprehensive studies involving congener-specific analysis of the mixtures tested would provide information regarding AR ligand-binding characteristics and capabilities of individual components of these complex mixtures. Future *in vitro* studies should examine the action of a wider range of congeners and their metabolites to comprehensively assess the interactions of specific PCBs with AR. The findings in these studies show that Aroclor 1254 exposure in murine systems through *in utero*, lactational, and oral exposure has a notable effect on prostate growth and development. As PCBs are present in all humans at detectable levels and their potential health impacts are far reaching, elucidating their mechanism of interaction with steroid hormone receptors will contribute to our understanding of the development of reproductive anomalies, impaired reproductive functions, endocrine related diseases, and androgen sensitive processes such as prostate function and prostate cancer.

Chapter VI – Summary and Discussion

Steroid hormones play critical roles in development and homeostasis particularly in the immune, neural and reproductive systems. Any disruption of their activity can have profound and lasting consequences. The identification of environmental contaminants able to interfere with hormonal function in the early 1990s has led to a flood of interest into the field of "endocrine disruption". This has resulted in a reassessment of the classical approach to toxicity testing that had previously relied upon endpoints of infertility and death, to develop new methodologies that are more sensitive to subtle changes produced by chronic as well as acute exposures. In addition there has been a growing awareness that many contaminants are persistent in the environment and readily accumulate in tissues such that over time an individual's body burden can exceed their exposure level many fold.

In recent years, a host of potential endocrine active compounds (EACs) have been identified, in particular many found to interact with ER, AhR and TR. Much of the early work in the field of endocrine active compounds has focussed upon potential ligands for ER and the development of assays to detect estrogenic activity. However, the structural relatedness of the steroid hormone superfamily members, particularly in the ligand-binding domain, logically suggests that if there are numerous environmental ligands for one then there are likely ligands for others.

I have developed an *in vitro* screen with which a range of compounds and mixtures were tested for their ability to interfere with receptor mediated gene transcriptional activation and ligand binding. For a few candidates, that produced interesting results in the *in vitro* reporter assay, *in vivo* studies in transgenic mice, which express a prostate specific androgen sensitive CAT reporter gene, were performed. To investigate whether activity *in vitro* correlates with *in vivo* effects upon health and development.

Of the three receptor systems tested *in vitro*, AR appeared to be the most responsive in the assays and further analysis was focussed there. This approach was also justified since this receptor has been relatively neglected by the endocrine disruptor field overall. The bias of our system towards maximising androgen sensitivity may explain why we saw many more responses with this receptor than with GR. For example prostate cell lines were used for both receptor assays. The prostate is known to be exquisitely sensitive to androgens but is not a particularly specialised target tissue for glucocorticoids. Of the 26 compounds tested that elicited a response, 16 were found to produce an antagonist effect with AR. In contrast, only Aroclor 1254 was able to produce an agonist response alone without the aid of DHT. Two further compounds, *p,p*'-DDD and hexachlorobenzene were also found to behave as AR agonists but only in an additive manner in combination with DHT. The predominance of antagonist activity can be rationalised. To antagonise the activity of a known ligand for a steroid receptor a compound can interfere with the normal action of the ligand at several points. Any interference with the synthesis, secretion, transport, uptake, activation, metabolism, receptor binding of steroid hormones or coregulator recruitment by receptor complex could manifest as antagonism. Whereas, to perform as an agonist a compound must mimic the endogenous or pharmacological ligand, bind to the receptor and induce an active conformation able to dimerise and interact with both the DNA and the transcriptional machinery.

6.1 Pesticides and Complex Mixtures

The array of pesticides tested on the three steroid hormone receptors are diverse in structure and produce correspondingly diverse responses. All but endosulfan appear to interact with AR, while only endosulfan, and *o,p*'-DDT interact with GR, and endosulfan, *p,p*'-DDE and *o,p*'-DDT interact with ER. Although all showed activity with at least one receptor type only *o,p*'-DDT affected all three producing an antagonist response with AR while enhancing the response to GR and ER in the presence of known agonist.

The first environmental contaminant shown to definitively affect AR activity was the DDT pesticide metabolite, *p,p*'-DDE [197, 198]. This compound was shown to act as a weak AR ligand, antagonizing transcriptional activity *in vitro* in the presence of DHT, and able to act as a weak AR agonist in the absence of DHT [201]. Following the restriction imposed on DDT production, the pesticide methoxychlor has largely replaced the use of DDT in North America. Methoxychlor and its metabolites however have also been shown to be AR antagonists *in vitro* in addition to their estrogenic activities [570, 571]. Metabolites of the fungicide vinclozolin have likewise been found to be potent AR-antagonists mediated through binding to AR [197, 343]. Administration of vinclozolin results in a host of permanent alterations to androgen-dependent tissues including decreased prostate weight [196, 572]. A structurally related fungicide, procymidone also binds AR and antagonizes DHT-induced transcriptional activity *in vitro* [573].

The analysis of the Fraser River sediments demonstrated potential endocrine activity in samples derived from a local environmental source. However, attempts to correlate this activity with the targeted composition analysis data (Appendix 1) demonstrate that our understanding of the endocrine potential of these samples is incomplete. In four of the sediments there were no detectable levels of known endocrine active compounds, as identified in this and other work. Nevertheless, two of these sediments did possess endocrine activity. This activity derives from EACs that were not quantified in the analysis that may be known but are likely as yet unidentified. Interestingly in both of these cases it was the GR assay that produced a response suggesting there might be an effective but as yet unidentified modulator for this receptor pathway in the environment at toxicologically relevant levels.

6.2 Alkylphenols

Alkylphenols have been characterised as weak estrogens [574]. Our work indicates that they may also be capable of disrupting the androgen axis although this effect may be indirect and not through binding to AR. In our assays nonylphenol was an agonist for ER as has been previously reported by others, but seemed able to antagonize AR mediated transactivation. However in the AR binding studies none of the alkylphenols tested demonstrated an ability to compete for the ligand-binding site. This is in agreement with a published study of NP binding with AR [203] in which the author proposes that NP may act by displacing androgens from transport proteins.

Despite this lack of apparent interaction with AR, the results of the mouse studies produced several intriguing physiological outcomes. Testis weight in NP-treated mice at eight weeks was significantly increased relative to controls and this correlated with an increased level of circulating thyroid hormone (T4) and increased CAT activity in treated animals. Though not statistically significant, there was a tendency for treated animals to have higher average testosterone levels than untreated animals.

Taken together these results suggest an enhancement of endogenous androgen action by NP. Although the compound is not capable of replacing androgens directly as is evident from the lack of response in the prostate of castrated adults and the apparent absence of AR binding, NP does appear to enhance responses to androgens in developing animals. The increase in CAT activity occurs in mice after puberty when testosterone levels naturally rise, again suggesting the NP enhances existing androgen rather than mimicking it.

The underlying mechanism by which NP may enhance androgen action is unclear but may be mediated through thyroid hormone, which has been shown to increase prostate weights in rats treated with T3 [439]. Although the same effect was not observed in adult males treated for 28 days with NP, this may indicate that the adult testis is less sensitive to alterations in thyroid status than the developing testis [430]. If NP induces an increase in thyroid hormone levels, which in turn affects Leydig cell differentiation and promotes androgen production, serum testosterone concentration could become elevated [428, 432, 575-578]. Alternatively, NP has been well established as a weak estrogen [203, 401], and at low doses estrogens can produce enlargement of the prostate in mice exposed fetally [579]. Furthermore, estrogen treatment can alter the AR level in the prostate of fetally exposed animals and neonatal imprinting by estrogens has been shown to permanently alter the sensitivity of the prostate to androgens [560, 580].

6.3 PCBs

PCBs are stable and persistent environmental contaminants that, due to their lipophilicity, migrate to fatty tissues and bioaccumulate in the food chain. The prostate is a highly lipophilic gland and likely accumulates many environmental contaminants, however no specific measurement of levels of PCB congeners have been documented. The action of PCBs and PCB metabolites as estrogens has been previously documented [574], however, the effects of PCBs on androgen mechanisms have not been thoroughly investigated. Our data clearly demonstrate the ability of PCBs to alter androgen-regulated processes *in vitro* at the molecular level and *in vivo*. The transactivation assay data demonstrates that Aroclor 1254 contains PCB congeners that can act as weak AR agonists in the absence of DHT. However, the majority of the PCB congeners and mixtures tested were antagonistic to AR activity in the presence of DHT. Interestingly the effects we have observed were almost exclusively in AR assays, suggesting PCBs interact relatively poorly with GR and ER.

All the Aroclor mixtures tested produced an antagonistic effect in the presence of DHT when cells were transfected with AR and competitively inhibit DHT from binding to AR in the competition studies. Together these results suggest that one or more specific congeners found in the Aroclor mixtures possess androgenic activity rather than representing a general property of all PCBs. Interestingly, Aroclors 1254 and 1260 did not elicit an identical response in either the androgen or glucocorticoid receptor, indicating that the individual congener make-up of a sample is of great importance in determining the endocrine activity. Different congeners can apparently have different effects upon a single receptor as has been previously reported [581-583]. The congeners tested in this work were selected primarily upon the basis of comparative analysis of their levels in two Aroclor mixtures. Since Aroclor 1254 exhibited androgenic activity in the cell culture assay whereas Aroclor 1260 did not we targeted congeners present at significantly higher levels in the former than the latter.

Previously, some hydroxylated PCB metabolites have been shown to bind to ARs in fish species [369] while our ligand displacement assay results show that specific PCB congeners can reduce natural ligand-binding to the mammalian AR. These results, combined with the ability of the Aroclors to alter the androgen driven luciferase reporter gene suggest that PCBs can affect AR activity in a wide range of species. As PCBs degrade extremely slowly, and highly chlorinated congeners remain in tissues for months or years before they are metabolised, an accumulation of these chemicals to levels at which androgenic activities occur seems probable.

Our results demonstrate that some PCBs may act as weak AR agonists even in the absence of DHT, and may antagonize AR activity in the presence of DHT. Since a number of PCB congeners are also known to activate AhR [562-565] it is plausible that AR may be antagonized indirectly by PCBs that serve as AhR ligands as well as antagonized directly by those PCBs which are AR ligands. Activation of AhR is known to interfere with the activity of AR and ER. Therefore complex mixtures of PCBs containing both AhR-ligands and AR or ER ligands may show suppression of transcriptional activity by multiple mechanisms. Furthermore, some congeners have been shown to alter AR protein levels in the prostate after developmental exposure and other congeners have been shown to increase the metabolism of androgens *in vivo*.

Since Aroclors are complex mixtures of dozens of PCBs, it is difficult to discern which specific congeners, or combination of congeners, are responsible for the results observed. The induction of transcription as well as the prevention of AR ligand binding may be due to the action of certain congeners on their own, or a combined effect of many components of the Aroclor mixtures. Our work with individual congeners goes some way to resolving this issue, but a more comprehensive screen with many more congeners will be required to clarify the structure activity relationships involved.

Structurally, PCB congeners can be subdivided into two broad classes on the basis of the orientation of the biphenyl rings as either co-planar or non-planar. In general, co-planar PCBs are more likely to interact with AhR and non-planar PCB congeners with chlorination in the ortho positions on the other hand may be more likely to interact with steroid receptors, as demonstrated for ER [584]. From our results, it appears that the non-planar orientation with two ortho substitutions, combined with one substitution at the meta and para position, respectively, binds AR with high affinity. This basic substitution pattern of congener 42 with the core of chlorine substitutions at 2, 2', 3 and 4' also seems to be predictive of the binding affinities of higher chlorinated congeners (PCBs 138 and 168). This may indicate that congeners with this basic substitution pattern seem to have an increased affinity for AR. This structure-activity relationship could possibly be used to establish which PCB congeners will exhibit the highest androgen modulating effects, presumably because of their strong interaction with AR.

6.4 Metabolism

Although environmental persistence and bioaccumulation are hallmarks of many of the contaminants identified as potential endocrine disrupters the potential for partial or total metabolism is an area of great interest. There is considerable and growing evidence to indicate that for some compounds the modifications they undergo within human or animal tissues can increase their toxic potential or modify their behaviour. For example hydroxylated PCBs have been shown to bind with higher affinity than their parent molecules to ER, TR and hormone transport proteins and methyl-sulphonated PCBs have been identified as a major class of human contaminant associated with toxicity in specific tissue types, such as the lung [585-587]. The metabolism of the compounds tested here was not investigated at length, although 2,2',3,4'-tetrachlorobiphenyl (PCB 42) would make an excellent target for hydroxylation based on Safe's criteria as stated earlier [456]. Furthermore there is the potential for modification of certain compounds to produce chemically unstable intermediates that are associated with injury to cellular components including DNA. In combination with the largely epigenetic and imprinting potential of endocrine pathways, any potential for free radical formation or other mutagenic behaviour would define that compound as a complete carcinogen. DES has been identified as one such candidate [588].

6.5 Structure Activity Relationships

As evidence for endocrine disruptors is compiled, and specific details (particularly binding properties) for each compound with various receptors are published, it should become possible to begin to assemble a structure-activity relationship scheme. Ultimately by improving our understanding of the minimal structural requirements for a ligand to interact with a given receptor we may be able to predict a compounds hazard potential to the endocrine system as a pseudohormone. Clearly this will not be a trivial undertaking since the multitude of receptor types, the latitude of their ligand-binding properties in different cellular contexts and the potential for metabolic modification of ligands must all be accounted for. In our study with PCBs we have identified a putative core structure, similar to PCB 42, which seems to promote binding to AR. Adding too many chlorine groups can lessen this interaction. Further targeted analysis of other structurally related congeners could clarify this model further.

A comparison of the structures of all of the compounds that interact with AR indicates substantial diversity. One universal feature is the presence of an unsaturated benzene ring in all the molecules. Of the chemicals tested only endosulfan lacks this motif and it fails to interact. In addition all of the pesticides are polychlorinated although the positioning of this substitution varies being on a side chain, the ring structure or both.

6.6 Experimental Methodology

The major method utilised throughout this work was the luciferase reporter gene assay. For both the AR and GR assay this method relies upon the use of prostate cell lines that may create a bias towards increased sensitivity to AR mediated mechanisms relative to GR effects. In addition, the reporter construct utilised in its native genomic context *in vivo* has been demonstrated to be responsive exclusively to AR and the acquisition of GR responsiveness results entirely from the manipulation of the receptor-binding portion of the promoter (Zhang et al., 2000). This androgenic bias of the assay may account for the fact that while many of the chemicals tested were found to interfere with androgen mediated gene transcription relatively few interfered with the corresponding glucocorticoid driven response. Similarly, the assay for interference with estrogen-mediated activity was substantially different using a different luciferase reporter construct transfected into a liver cell line (HepG2) because the prostate cells were relatively unresponsive to estrogens. The ER assay was markedly less sensitive because it had a higher background and smaller response range (less than 10 fold). Therefore, although the data presented here might suggest that AR is more sensitive to endocrine interference by environmental contaminants, this is not necessarily the case.

Data from cell culture assays contains considerable variability. The level of luciferase measured in the luminescence assay was found to vary by as much as 10 fold between similar experiments. The reasons for this variation are many but it appears that the exact culture conditions of the cells and timing are critical to reproducibility of results. Factors such as cell passage number, confluency prior to seeding onto plates for an experiment, and in particular confluence levels at the time of transfection and the addition of test compounds, had the greatest effects. It is not entirely practicable to control for all such variables, and in most cases it is the magnitude of response that varies and not the pattern (agonist, antagonist or no effect).

I have made use of a battery of assays to test for endocrine activity amongst the compounds and mixtures investigated. As is evident from my own results and from the literature in this field the results of a single assay cannot be treated as reliable evidence for the potential activity of a compound, particularly considering the health and regulatory implications reports may have. It is important to be aware of the limitations and specificity of any one assay, and the potential for dramatic differences in sensitivity of one system compared to another. Fortunately the steroid receptors themselves are highly conserved amongst mammalian species. For example the sequences of the human and rat receptors are identical in the ligand-binding domain of AR. Nevertheless interspecies differences can still result from numerous other variables including co-regulators, developmental timing and metabolic competence.

This dissertation describes the development of an *in vitro* screen used to identify several novel endocrine active compounds that interact with AR, GR or ER. The use of competitive binding analyses determined that several of these compounds interact directly with AR. Furthermore three EACs that interfered with androgen activity in the *in vitro* screen were tested in an in *vivo* transgenic mouse model. All three, nonylphenol, Aroclor 1254 and PCB congener 42 were found to produce physiological effects although only Aroclor 1254 indicated a clear interaction with AR *in vivo*. Comparison of the structures of the contaminants tested that were found to interact with AR identified that polychlorination and an unsaturated benzene ring as common structural motifs. Furthermore amongst the PCB congeners tested those with a 2,3,2',4' core substitution pattern were all competitors with known ligands for the AR-LBD.

The focus of the research presented has dealt with endocrine disruption via ligand mimicry. Numerous alternative pathways are equally likely to contribute to the toxicity of environmental chemicals. Interference at any point in a hormonal pathway, be it the synthesis, release, transport, uptake, or receptor binding of the hormone itself, or modulation of the cellular effecter, or hormone metabolism will impact endocrine homeostasis. It is the very complexity of the endocrine system and the multitude of targets for disturbance that have caused such concern over the health implications of environmental contaminants and advocate caution in the use and disposal of mankind's chemical arsenal.

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PCB Coplanars (Fall 1996)- data incomplete

Sample ID	96LCH1	96LCH4	96LCH5	96LCH6	96LCH6d	96MAN1	96MAN2	96MAN3	96MAN4	96NAR1	96NAR2	96NAR3	96NAR3d	96NAR4
PCB #77 (3,3',4,4' TCB)	0.8	-0.6	-0.6	0.65	-0.6	3.7	2.4	35	5.1	14				
PCB #126 (3,3',4,4',5 PCB)	-0.3	-0.3	-0.3	-0.3	-0.3	0.32	-0.3	1	0.31	0.78				
PCB #169 (3,3',4,4',5,5' HCI	-0.4	-0.4	-0.4	-0.4	-0.4	-0.4	-0.4	-0.4	-0.4	-0.7				
% Surrogate Recovery														
13C-PCB #77	67	70	79	75	66	69	64	69	66	70				
13C-PCB #126	70	81	82	79	75	71	64	71	73	72				
13C-PCB #169	65	83	79	77	71	66	63	70	68	69				

Values expressed as pg/g dry weight.
Values below detection limits shown as negative
Lab duplicates denoted by a "d" following the sample ID.
Values are recovery corrected.

Appendix 1

PCB Aroclors (Fall 1996)

Sample ID	96LCH1	96LCH4	96LCH5	96LCH6	96LCH6d	96MAN1	96MAN2	96MAN3	96MAN4	96NAR1	96NAR2	96NAR3	96NAR4
Aroclor 1242	-0.18	-0.15	-0.3	-0.11	-0.12	-0.33	-0.56	3.4	-0.57	-0.62	-3.9	1	-5
Aroclor 1254	-0.22	-0.27	-0.24	-0.18	-0.18	-0.61	-0.38	3.7	-0.69	1.7	4.1	0.94	0.73
Aroclor 1260	-0.23	-0.21	-0.24	-0.17	-0.2	-0.55	-0.45	-1.6	-0.57	-1	9.3	-0.55	0.4

Values expressed as ng/g dry weight.
Values below detection limits shown as negative.
Lab duplicates denoted by a "d" following the sample ID.

PCB Congeners in Bed Sediment (1996)

Sample ID	96LCH1	96LCH4	96LCH5	96LCH6	96LCH6d	96MAN1	96MAN2	96MAN3	96MAN4	96NAR1	96NAR2	96NAR3	96NAR4
8/5	-0.007	-0.005	-0.008	-0.005	-0.006	-0.01	0.06	0.07	-0.03	-0.06	0.16	0.02	0.04
15	-0.03	-0.11	-0.01	-0.006	-0.008	0.03	-0.03	0.1	0.08	0.12	0.13	0.05	-0.04
19	-0.01	-0.009	-0.02	-0.007	-0.007	-0.02	-0.03	-0.05	-0.03	-0.38	-0.14	-0.04	-0.31
18	-0.01	-0.009	-0.02	-0.007	-0.007	-0.02	-0.03	0.12	-0.03	-0.38	-0.24	-0.04	-0.31
17	-0.01	-0.009	-0.02	-0.007	-0.007	-0.02	0.09	0.07	-0.03	-0.38	-0.24	-0.04	-0.31
24/27	-0.01	-0.009	-0.02	-0.007	-0.007	-0.02	-0.03	-0.05	-0.03	-0.38	-0.24	-0.04	-0.31
16/32	-0.01	-0.009	-0.02	-0.007	-0.007	-0.02	-0.03	0.08	-0.03	-0.38	-0.24	-0.04	-0.31
26	-0.009	0.02	-0.01	-0.005	0.01	-0.01	-0.03	-0.07	-0.03	-0.3	-0.19	-0.03	-0.24
25	-0.009	-0.007	-0.01	-0.005	-0.006	-0.01	-0.03	-0.04	-0.03	-0.3	-0.19	-0.03	-0.24
31/28	0.03	0.01	-0.01	0.01	-0.007	0.07	0.08	0.61	0.08	-0.3	0.48	0.19	-0.24
33	0.03	0.02	-0.01	-0.005	-0.006	-0.03	-0.03	0.13	-0.05	-0.3	-0.19	0.05	-0.24
22	-0.009	-0.007	-0.01	-0.005	-0.006	-0.01	-0.03	-0.08	-0.03	-0.3	-0.19	-0.03	-0.24
45	-0.02	-0.02	-0.02	-0.009	-0.01	-0.06	-0.01	-0.08	-0.07	-0.03	0.02	-0.02	-0.02
46	-0.02	-0.02	-0.02	-0.009	-0.01	-0.06	-0.01	-0.08	-0.07	-0.03	-0.02	-0.02	-0.02
52	-0.02	-0.02	-0.02	-0.009	-0.01	-0.06	0.05	0.42	-0.07	0.16	0.24	0.09	0.06
49	-0.02	-0.02	-0.02	-0.008	-0.008	-0.05	0.02	0.28	-0.07	0.08	0.12	0.04	0.02
47/48	-0.02	-0.02	-0.02	-0.008	-0.008	-0.05	0.02	0.18	-0.06	0.04	0.09	-0.02	-0.01
44	-0.03	-0.02	-0.02	-0.01	-0.01	-0.06	0.03	0.37	-0.08	0.08	0.13	0.06	0.03
42	-0.03	-0.02	-0.02	-0.01	-0.01	-0.06	-0.01	-0.11	-0.08	-0.03	0.06	-0.02	-0.02
41/71/64	-0.04	-0.05	-0.04	-0.01	-0.01	-0.06	0.03	0.57	-0.08	0.11	0.16	0.12	0.03
40	-0.02	-0.02	-0.02	-0.01	-0.01	-0.06	-0.01	-0.08	-0.08	-0.03	-0.02	-0.02	-0.02
74	0.06	0.06	-0.05	-0.01	-0.01	-0.06	0.02	0.48	-0.08	0.06	0.1	0.11	0.03
70/76	-0.02	-0.05	-0.03	-0.01	-0.01	-0.06	0.04	0.67	-0.08	0.16	0.25	0.11	0.07
66	-0.02	-0.04	-0.03	-0.01	-0.009	-0.04	0.03	0.47	-0.05	0.09	0.16	0.11	0.04
56/60	0.04	-0.04	-0.01	-0.007	-0.007	-0.04	0.01	0.33	-0.05	0.05	0.11	0.04	0.02
95	-0.007	-0.009	-0.008	-0.006	-0.006	-0.03	0.03	0.2	-0.03	0.13	0.36	0.08	0.05
91	-0.007	-0.009	-0.008	-0.006	-0.006	-0.02	-0.01	0.05	-0.02	-0.04	0.04	-0.02	-0.01
84/89	-0.007	-0.009	-0.008	-0.006	-0.006	-0.02	-0.01	0.12	-0.02	-0.06	0.14	0.03	0.03
101/90	-0.007	-0.009	-0.008	-0.006	-0.006	0.03	0.04	0.29	0.03	0.18	0.54	0.09	0.07
99	-0.007	-0.009	-0.008	-0.006	-0.006	-0.02	-0.01	0.15	-0.02	0.07	0.15	0.04	0.03
83	-0.008	-0.01	-0.008	-0.006	-0.006	-0.02	-0.01	-0.05	-0.02	-0.04	-0.02	-0.02	-0.02
97	-0.008	-0.01	-0.008	-0.006	-0.006	-0.02	-0.01	0.11	-0.02	-0.04	0.1	0.03	0.02
87	-0.008	-0.01	-0.008	-0.006	-0.006	-0.02	-0.01	0.12	-0.02	-0.07	0.17	0.03	0.03
85	-0.008	-0.01	-0.008	-0.006	-0.006	-0.02	-0.01	0.07	-0.02	-0.04	0.06	-0.02	-0.02
110	-0.008	-0.01	-0.008	-0.006	-0.006	0.07	0.04	0.45	0.06	0.22	0.62	0.16	0.1
107	-0.006	-0.008	-0.007	-0.005	-0.005	-0.02	-0.01	-0.04	-0.02	-0.03	0.03	-0.02	-0.01
118	0.01	-0.007	-0.006	-0.005	-0.005	0.05	0.03	0.27	-0.05	0.13	0.31	0.09	0.05
114	-0.006	-0.008	-0.007	-0.005	-0.005	-0.02	-0.01	-0.04	-0.02	-0.03	-0.01	-0.02	-0.01
105	-0.006	-0.008	-0.007	-0.005	-0.005	-0.02	-0.01	0.1	-0.02	0.05	0.12	0.04	0.02
136	-0.02	-0.01	-0.01	-0.008	-0.01	-0.04	-0.01	-0.06	-0.04	-0.04	0.25	-0.03	-0.02
151	-0.02	-0.01	-0.01	-0.008	-0.01	-0.04	-0.01	-0.06	-0.04	-0.04	0.35	-0.03	0.02
144/135	-0.02	-0.01	-0.01	-0.008	-0.01	-0.04	-0.01	-0.06	-0.04	-0.04	0.21	-0.03	-0.02
149	-0.02	-0.01	-0.01	-0.008	-0.01	-0.04	0.02	0.11	-0.04	0.17	1.1	0.08	0.07
134	-0.02	-0.01	-0.01	-0.008	-0.01	-0.04	-0.01	-0.06	-0.04	-0.04	0.05	-0.03	-0.02
131	-0.02	-0.01	-0.01	-0.008	-0.01	-0.04	-0.01	-0.06	-0.04	-0.04	-0.02	-0.03	-0.02
146	-0.005	-0.003	-0.004	-0.002	-0.003	-0.01	-0.004	-0.02	-0.04	-0.01	0.06	-0.03	-0.007
153	-0.01	-0.009	-0.01	-0.006	-0.008	0.04	0.02	0.12	0.04	0.13	0.89	0.1	0.07

PCB Congeners in Bed Sediment (1996)

Sample ID	96LCH1	96LCH4	96LCH5	96LCH6	96LCH6d	96MAN1	96MAN2	96MAN3	96MAN4	96NAR1	96NAR2	96NAR3	96NAR4
141	-0.02	-0.01	-0.02	-0.009	-0.01	-0.04	-0.02	-0.07	-0.04	-0.04	0.23	-0.04	0.02
130	-0.02	-0.01	-0.01	-0.008	-0.01	-0.04	-0.01	-0.07	-0.04	-0.04	-0.02	-0.03	-0.02
137	-0.02	-0.01	-0.01	-0.008	-0.01	-0.04	-0.01	-0.07	-0.04	-0.04	-0.02	-0.03	-0.02
138/163/164	-0.02	-0.01	-0.01	-0.008	-0.01	0.05	0.03	0.18	0.05	0.17	1.1	0.15	0.1
158	-0.02	-0.01	-0.01	-0.008	-0.01	-0.04	-0.01	-0.07	-0.04	-0.04	0.1	-0.03	-0.02
129	-0.02	-0.01	-0.01	-0.008	-0.01	-0.04	-0.01	-0.07	-0.04	-0.04	-0.02	-0.03	-0.02
128	-0.02	-0.01	-0.02	-0.009	-0.01	-0.04	-0.02	-0.07	-0.04	-0.04	0.09	-0.03	-0.02
156	-0.02	-0.01	-0.01	-0.008	-0.01	-0.04	-0.01	-0.07	-0.04	-0.03	0.08	-0.03	-0.02
157	-0.02	-0.01	-0.01	-0.008	-0.01	-0.04	-0.01	-0.07	-0.04	-0.03	-0.02	-0.03	-0.02
179	-0.01	-0.01	-0.01	-0.008	-0.09	-0.02	-0.02	-0.07	-0.02	-0.05	0.21	-0.03	-0.02
176	-0.01	-0.01	-0.01	-0.008	-0.09	-0.02	-0.02	-0.07	-0.02	-0.05	0.06	-0.03	-0.02
178	-0.01	-0.01	-0.01	-0.008	-0.09	-0.02	-0.02	-0.07	-0.02	-0.05	0.06	-0.03	-0.02
175	-0.01	-0.01	-0.01	-0.008	-0.09	-0.02	-0.02	-0.07	-0.02	-0.05	-0.04	-0.03	-0.02
187/182	-0.01	-0.01	-0.01	-0.008	-0.09	-0.02	-0.02	-0.07	-0.02	-0.05	0.44	-0.03	0.02
183	-0.01	-0.01	-0.01	-0.01	-0.01	-0.03	-0.02	-0.09	-0.03	-0.06	0.26	-0.04	-0.02
185	-0.01	-0.01	-0.01	-0.01	-0.01	-0.03	-0.02	-0.09	-0.03	-0.06	0.06	-0.04	-0.02
174	-0.01	-0.01	-0.01	-0.01	-0.01	-0.03	-0.02	-0.09	-0.03	-0.06	0.43	-0.04	-0.02
177	-0.01	-0.01	-0.01	-0.01	-0.01	-0.03	-0.02	-0.09	-0.03	-0.06	0.23	-0.04	-0.02
171	-0.01	-0.01	-0.01	-0.01	-0.01	-0.03	-0.03	-0.09	-0.03	-0.06	0.1	-0.04	-0.02
172	-0.01	-0.01	-0.01	-0.01	-0.01	-0.03	-0.02	-0.09	-0.03	-0.06	0.05	-0.04	-0.02
180	-0.01	-0.01	-0.01	-0.01	-0.01	-0.03	-0.02	-0.09	-0.03	0.08	0.94	0.08	0.05
193	-0.01	-0.01	-0.01	-0.01	-0.01	-0.03	-0.02	-0.09	-0.03	-0.06	0.05	-0.03	-0.02
191	-0.01	-0.01	-0.01	-0.01	-0.01	-0.03	-0.02	-0.09	-0.03	-0.06	-0.04	-0.03	-0.02
170/190	-0.02	-0.02	-0.02	-0.01	-0.02	-0.04	-0.03	-0.12	-0.04	-0.08	0.53	-0.04	-0.02
189	-0.02	-0.02	-0.02	-0.01	-0.02	-0.04	-0.03	-0.12	-0.04	-0.08	-0.06	-0.03	-0.02
201	-0.01	-0.008	-0.01	-0.007	-0.01	-0.02	-0.02	-0.07	-0.02	-0.08	-0.02	-0.06	-0.02
197	-0.02	-0.02	-0.02	-0.02	-0.02	-0.05	-0.05	-0.16	-0.06	-0.19	-0.04	-0.06	-0.04
198	-0.02	-0.02	-0.02	-0.02	-0.02	-0.05	-0.05	-0.16	-0.06	-0.19	-0.04	-0.06	-0.04
199	-0.02	-0.02	-0.02	-0.02	-0.02	-0.05	-0.05	-0.16	-0.06	-0.19	0.24	0.08	-0.04
196/203	-0.02	-0.02	-0.02	-0.02	-0.02	-0.04	-0.04	-0.14	-0.05	-0.17	0.24	-0.05	-0.04
195	-0.02	-0.02	-0.02	-0.02	-0.02	-0.04	-0.04	-0.14	-0.05	-0.17	0.06	-0.06	-0.04
194	-0.03	-0.02	-0.03	-0.02	-0.03	-0.06	-0.06	-0.19	-0.07	-0.23	0.2	-0.06	-0.04
205	-0.03	-0.02	-0.03	-0.02	-0.03	-0.06	-0.06	-0.19	-0.07	-0.23	-0.04	-0.06	-0.04
208	-0.02	-0.02	-0.02	-0.02	-0.02	-0.03	-0.06	-0.12	-0.03	-0.13	-0.03	-0.08	-0.02
207	-0.02	-0.02	-0.02	-0.02	-0.02	-0.03	-0.06	-0.12	-0.03	-0.13	-0.03	0.14	-0.02
206	-0.02	-0.02	-0.02	-0.02	-0.02	-0.03	-0.06	-0.12	-0.03	-0.13	-0.03	-0.08	-0.02
209	-0.01	-0.01	-0.02	-0.01	-0.01	-0.02	-0.05	-0.09	-0.02	-0.17	-0.02	0.24	-0.02

Values expressed as ng/g dry weight.
Values below detection limits shown as negative
Lab duplicates denoted by a "d" following the sample ID.
Values recovery corrected

Appendix 1

Dioxins in Bed Sediment (1996)

Sample ID	96LCH1	96LCH4	96LCH5	96LCH6	96MAN1	96MAN2	96MAN3	96MAN4	96NAR1	96NAR2	96NAR2d	96NAR3	96NAR4
T4CDD (TOTAL)	1	0.5	0.8	0.4	2.7	0.9	2.9	3	4.4	6.2	6.5	2.1	6.4
2,3,7,8 TCDD	-0.1	-0.1	-0.1	-0.1	0.1	0.1	0.1	0.1	0.2	0.3	0.3	0.1	0.3
P5CDD (TOTAL)	0.5	0.7	0.4	0.2	2.3	0.3	2.5	3.4	3.5	11	9.9	2.9	7.6
1,2,3,7,8 PCDD	-0.1	-0.1	-0.1	-0.1	0.2	-0.2	0.2	0.3	0.4	1.1	1.1	0.3	0.6
H6CDD (TOTAL)	3.8	3.2	2.5	1.8	10	6.5	13	18	38	78	74	20	33
1,2,3,4,7,8 H6CDD	-0.2	-0.2	-0.2	-0.2	-0.3	-0.3	0.3	0.5	0.6	1.5	1.5	0.4	0.8
1,2,3,6,7,8 H6CDD	0.2	0.2	-0.2	-0.2	1.1	0.7	1.2	1.9	5.4	13	13	3.2	5.2
1,2,3,7,8,9 H6CDD	0.4	0.3	0.4	0.2	0.9	0.6	1.1	1.7	2.6	5.3	5.1	1.7	2.6
H7CDD (TOTAL)	8.3	9	7.1	6.2	30	14	32	54	76	190	180	56	92
1,2,3,4,6,7,8 H7CDD	3.7	3.4	2.6	2.4	12	5.8	13	22	35	100	97	25	43
O8CDD (TOTAL)	44	44	31	29	100	46	120	160	240	660	590	180	370
T4CDF (TOTAL)	0.6	0.3	0.3	0.1	2.6	1.3	2	2.2	4.8	11	11	3	7.7
2,3,7,8 T4CDF	0.3	0.2	0.2	0.1	0.9	0.7	0.7	0.7	1.5	2.2	2.3	0.9	2.7
P5CDF (TOTAL)	1	-0.1	-0.1	-0.1	1.3	0.5	1.5	2.3	6.4	31	30	5.2	9.6
1,2,3,7,8 P5CDF	0.2	-0.1	-0.1	-0.1	-0.2	-0.2	-0.2	-0.2	0.2	0.5	0.4	-0.2	0.2
2,3,4,7,8 P5CDF	0.1	0.1	-0.1	-0.1	-0.2	-0.2	-0.2	-0.2	0.3	0.7	0.7	-0.2	0.4
H6CDF (TOTAL)	0.3	-0.2	-0.2	-0.2	4.5	1.5	3.9	5.3	20	99	94	20	34
1,2,3,4,7,8 H6CDF	-0.2	-0.2	-0.2	-0.2	-0.3	-0.3	-0.3	-0.3	0.5	2	2	0.5	0.9
1,2,3,6,7,8 H6CDF	-0.2	-0.2	-0.2	-0.2	-0.3	-0.3	-0.3	-0.3	0.5	2.1	2	0.4	0.6
2,3,4,6,7,8 H6CDF	-0.2	-0.2	-0.2	-0.2	-0.3	-0.3	-0.3	-0.3	0.5	2.1	2	0.5	0.6
1,2,3,7,8,9 H6CDF	-0.2	-0.2	-0.2	-0.2	-0.3	-0.3	-0.3	-0.3	-0.4	-0.4	-0.4	-0.2	-0.3
H7CDF (TOTAL)	0.6	0.6	-0.4	0.6	7.6	2.8	7.3	12	38	160	160	41	65
1,2,3,4,6,7,8 H7CDF	-0.4	-0.4	-0.4	-0.4	2.6	1.1	2.7	4.3	13	59	59	13	21
1,2,3,4,7,8,9 H7CDF	-0.4	-0.4	-0.4	-0.4	-0.6	-0.4	-0.4	-0.6	0.7	2.6	3.1	0.8	1.3
O8CDF (TOTAL)	0.9	1.2	-0.6	1.3	6.2	1.3	3.8	6.8	17	57	66	17	27
2,3,7,8-TCDD TEQs	0.2	0.1	0.1	0.1	0.7	0.4	0.8	1.2	2.5	6.4	6.3	1.6	3.2

Values expressed as pg/g dry weight.
Values below detection limits shown as negative
Lab duplicates denoted by a "d" following the sample ID.
Values are recovery corrected.

Appendix 1

Dioxins in Bed Sediment (1996)

Sample ID	96LCH1	96LCH4	96LCH5	96LCH6	96MAN1	96MAN2	96MAN3	96MAN4	96NAR1	96NAR2	96NAR2d	96NAR3	96NAR4
% Surrogate Recovery													
13C-2,3,7,8 T4CDF	85	51	70	68	81	92	86	110	100	100	94	91	73
13C-2,3,7,8 T4CDD	98	65	94	74	89	100	79	110	88	90	88	85	80
13C-1,2,3,7,8 P5CDF	89	46	67	62	78	87	63	86	76	80	80	68	55
13C-1,2,3,7,8 P5CDD	100	42	67	64	78	86	64	90	99	84	86	71	60
13C-1,2,3,4,7,8 H6CDF	80	46	68	60	67	110	82	97	88	81	75	68	71
13C-1,2,3,4,7,8 H6CDD	96	50	84	67	83	98	79	100	85	83	78	77	85
13C-1,2,3,4,6,7,8 H7CD	100	43	77	62	73	78	65	74	74	71	65	58	66
13C-1,2,3,4,6,7,8 H7CD	120	33	70	55	68	80	66	77	73	72	67	63	69
13C-O8CDD	95	25	69	53	63	69	60	67	76	87	82	79	85

Values expressed as pg/g dry weight.
Values below detection limits shown as negative
Lab duplicates denoted by a "d" following the sample ID.
Values are recovery corrected.

Appendix 1

4-Nonylphenol and Bisphenol A in Bed Sediments from the Fraser Basin (1996)

Sample ID	96LCH1	96LCH4	96LCH4d	96LCH5	96LCH6	96MAN1	96MAN2	96MAN3	96MAN4	96NAR1	96NAR2	96NAR3	96NAR3d	96NAR4
TOTAL 4-NONYLPHENOL	5.8	-5	-5	-5	-5	8.9	7.4	15	13	30	47	19	22	52
BISPHENOL-A	-1.8	-1.6	-1.6	-2	-1.6	-2.5	-2.5	-3	-2	7	13	3.7	19	4.1
% Surrogate Recovery														
d6-Bisphenol-A	78	86	87	78	83	71	75	74	78	89	73	83	70	74

Values expressed as ng/g dry weight.
Values below detection limits shown as negative
Lab duplicates denoted by a "d" following the sample ID.
Reruns denoted by an "r" following the sample ID
Concentrations are not recovery corrected.

Appendix 1

PAHs in Bed Sediment (Fall 1996)

Sample ID	96LCH1	96LCH4	96LCH5	96LCH6	96MAN1	96MAN1d	96MAN2	96MAN3	96MAN4	96NAR1	96NAR2	96NAR3	96NAR3d	96NAR4
Naphthalene	1.9	1.4	2	1.2	5	5.3	2	7.8	5.3	12	19	17	11	8.3
Acenaphthylene	-0.09	-0.19	-0.14	-0.18	1	1.6	0.34	1.6	0.56	1.9	4	1.7	1.3	0.83
Acenaphthene	-0.35	-0.38	-0.36	-0.39	1.6	1.7	-0.42	2.3	3.6	4.2	17	13	6.2	2.7
Fluorene	1	0.65	0.49	0.62	4	4.2	1.6	5.1	7.3	10	25	13	9.2	6.8
Phenanthrene	4.2	2.7	3.2	2.2	16	20	5.8	20	54	38	90	40	26	25
Anthracene	0.28	0.27	0.2	0.08	2.8	2.8	0.72	2.4	5.3	7	18	6.7	5.2	3.3
Fluoranthene	3	2.5	3	0.78	20	25	6.3	24	100	62	170	50	49	39
Pyrene	3	2.4	3.3	0.91	16	20	5.6	21	79	49	140	42	42	34
Benz(a)anthracene	1.1	0.71	1.5	0.18	7.1	8.6	1.6	6.7	29	19	54	18	18	13
Chrysene	2.4	1.5	2.7	0.7	18	13	3.6	12	51	32	87	30	24	23
Benzofluoranthenes	2.6	1.4	1.6	0.36	15	16	3.4	13	66	38	100	45	32	28
Benzo(e)pyrene	1.1	0.73	0.84	0.26	6.4	7.3	1.4	6	26	16	45	18	15	13
Benzo(a)pyrene	1.1	0.62	0.88	-0.16	6.1	7.7	1.4	6	38	20	51	17	17	13
Perylene	18	5.1	12	3.2	33	33	26	51	36	38	62	26	25	79
Dibenz(ah)anthracene	0.2	0.12	-0.16	-0.1	0.77	1.1	-0.22	0.8	4.9	2.5	6.4	2.6	2.1	-2.2
Indeno(1,2,3-cd)pyrene	0.91	0.71	0.5	0.16	4.6	5.7	1.4	4.3	27	17	37	14	12	12
Benzo(ghi)perylene	1.5	0.98	0.91	0.39	6.3	7	2	6.1	28	15	42	15	14	14
C1 naphthalenes	4.1	1.7	4.2	2.2	8.2	8.2	3	8.2	7.7	17	28	24	11	11
C2 naphthalenes	3.6	-0.66	2.9	1.2	7.4	9	1.2	7.9	8	30	41	20	9.7	12
C3 naphthalenes	2.9	2.8	2.1	2	6.8	4.4	2.3	8.9	3.3	33	79	10	9.6	13
C4 naphthalenes	-0.73	-0.79	-0.77	-0.81	-1	-1	-0.86	4.6	-0.8	42	100	-0.84	-0.83	-1
C1 phen,anth	5.1	3.8	3.9	2.5	15	16	5.5	17	27	54	110	23	18	23
C2 phen,anth	7.9	3.2	7.6	2.7	24	24	9.2	30	41	99	310	37	30	40
C3 phen,anth	-0.11	2.1	-0.12	-0.34	12	10	3	21	24	100	280	24	22	27
C4 phen,anth	65	12	40	8.2	26	23	13	110	76	140	310	32	68	93
Retene	65	12	40	8.2	26	23	13	110	76	95	310	32	68	93
Dibenzothiophene	-0.36	-0.38	-0.38	-0.38	1.2	1.3	0.48	1.5	3	3.4	7.8	3.4	2.2	1.6
C1 dibenzothiophene	0.42	-0.11	0.44	-0.1	1.6	1.6	0.48	2.6	2.2	5.8	16	2.5	2.2	2.5
C2 dibenzothiophene	0.29	0.06	-0.05	-0.06	2.2	2.5	0.72	2.4	3.6	16	36	2.2	3.2	4.3

Values expressed as ng/g dry weight.
Values below detection limits shown as negative

Appendix 1

Lab duplicates denoted by a "d" following the sample ID.
Values recovery corrected.

PAHs in Bed Sediment (Fall 1996)

Sample ID	96LCH1	96LCH4	96LCH5	96LCH6	96MAN1	96MAN1d	96MAN2	96MAN3	96MAN4	96NAR1	96NAR2	96NAR3	96NAR3d	96NAR4
% Surrogate Recovery														
Naphthalene d-8	73	64	69	65	63	75	71	73	70	24	48	62	55	53
Acenaphthene d-10	81	73	77	74	76	81	80	76	80	39	58	78	68	69
Phenanthrene d-10	89	82	82	86	84	85	89	83	88	64	67	90	92	83
Pyrene d-10	92	87	85	91	89	89	92	85	91	70	58	88	87	75
Chrysene d-12	85	85	73	94	74	74	81	67	76	73	40	65	73	52
Benzo(a)pyrene d-12	110	99	82	110	97	100	100	91	100	79	66	91	97	75
Perylene-d12	96	89	72	99	88	92	94	82	94	77	61	83	89	68
Dibenz(ah)anthracene d-14	79	74	55	86	71	75	83	65	78	72	61	80	78	56
Benzo(ghi)perylene d-12	79	72	57	82	72	77	81	70	82	59	62	81	79	57
2-Methylnaphthalene d-10	66	60	63	59	62	69	66	66	66	26	48	63	52	54

Values expressed as ng/g dry weight.
Values below detection limits shown as negative
Lab duplicates denoted by a "d" following the sample ID.
Values recovery corrected.

Appendix 1

Pesticides in Bed Sediment (Fall 1996)

Sample ID	96LCH1	96LCH4	96LCH5	96LCH6	96LCH6d	96MAN1	96MAN2	96MAN3	96MAN4	96NAR1	96NAR2	96NAR3	96NAR4
Hexachlorobenzene (284)	0.05	0.05	-0.05	-0.05	0.04	0.09	0.06	0.08	-0.08	0.15	0.18	0.06	0.09
alpha HCH (219)	-0.09	-0.03	-0.07	-0.03	-0.05	0.25	-0.07	-0.27	-1	-0.17	-0.13	-0.13	-0.09
beta HCH (219)	0.4	0.6	0.2	-0.1	-0.11	1	0.54	-0.49	-2	0.79	-0.23	0.26	1.1
gamma HCH (219)	0.27	0.39	0.2	-0.1	-0.07	0.84	-0.11	-0.41	-1.7	-0.26	-0.2	0.55	0.17
delta HCH (219)	-0.13	-0.07	0.1	-0.05	-0.07	-0.31	-0.11	-0.2	-1.7	-0.26	-0.2	-0.19	-0.14
Heptachlor (337)	-0.1	-0.06	-0.11	-0.06	-0.07	-0.23	-0.2	-0.63	-0.15	-0.77	-0.28	-0.13	-0.18
Aldrin (263)	-0.02	-0.02	-0.02	-0.04	-0.03	-0.04	-0.05	-0.11	-0.03	-0.14	-0.13	-0.19	-0.08
Oxychlordane (373)	0.37	-0.1	0.38	-0.1	-0.12	-0.25	-0.31	0.96	-0.19	-0.9	-1.1	-0.2	-0.55
trans-Chlordane (373)	-0.02	-0.009	-0.01	-0.01	-0.01	-0.02	-0.04	-0.08	-0.02	-0.08	0.24	0.03	-0.03
cis-Chlordane (373)	-0.02	-0.01	-0.02	-0.01	-0.02	-0.02	-0.04	-0.11	-0.02	-0.1	0.21	0.03	-0.04
p,p'-DDE (246)	0.06	-0.03	0.03	-0.03	-0.04	0.3	-0.02	0.23	0.15	0.42	0.97	0.18	0.42
trans-Nonachlor (409)	-0.01	-0.008	-0.01	-0.008	-0.009	-0.02	-0.03	-0.07	-0.02	-0.08	0.19	-0.02	-0.03
p,p'-DDD (235)	0.09	0.09	-0.02	-0.02	-0.03	0.36	0.16	0.29	0.35	0.43	0.83	0.4	0.89
o,p'-DDT (235)	-0.02	-0.02	-0.03	-0.02	-0.02	-0.03	-0.04	0.63	-0.04	-0.1	0.23	0.2	-0.07
p,p'-DDT (235)	2.1	1.2	1.5	0.26	0.25	3.7	0.35	4.7	7	1.8	3	4	2.9
Mirex (272)	-0.11	0.14	0.15	-0.1	-0.1	0.18	-0.06	-0.24	-0.13	-0.14	-0.1	-0.15	-0.07
Heptachlor Epoxide	-0.1	-0.08	-0.03	-0.04	-0.05	-0.12	-0.05	-0.04	-0.05	-0.13	-0.07	-0.03	-0.06
alpha-Endosulphan (I)	-0.05	-0.04	-0.02	-0.02	-0.03	-0.06	-0.03	-0.02	-0.03	-0.1	-0.05	-0.02	-0.04
Dieldrin	-0.1	-0.08	-0.03	-0.04	-0.05	-0.12	-0.05	-0.04	-0.06	0.5	-0.07	-0.03	-0.06
Endrin	-0.26	-0.19	-0.07	-0.09	-0.12	-0.28	-0.12	-0.09	-0.13	-0.32	-0.16	-0.07	-0.14
beta-Endosulphan (II)	-0.06	-0.05	-0.02	-0.03	-0.04	-0.07	-0.03	-0.02	-0.11	-0.09	-0.06	-0.03	-0.06
Endosulphan Sulphate	-0.07	-0.06	-0.02	-0.03	-0.05	-0.11	-0.04	-0.03	-0.06	-0.12	-0.08	-0.03	-0.07
Methoxychlor	-0.46	-0.42	-0.15	-0.21	-0.29	-0.62	-0.27	-0.2	-0.29	-1.2	-0.39	-0.17	-0.33

Values expressed as ng/g dry weight.
Values below detection limits shown as negative
Lab duplicates denoted by a "d" following the sample ID.
Values are recovery corrected.

Appendix 1

Pesticides in Bed Sediment (Fall 1996)

Sample ID	96LCH1	96LCH4	96LCH5	96LCH6	96LCH6d	96MAN1	96MAN2	96MAN3	96MAN4	96NAR1	96NAR2	96NAR3	96NAR4
% Surrogate Recovery													
d4-alpha-Endosulphan	66	64	66	69	62	58	54	69	76	65	70	58	59
13C6-Hexachlorobenzene (29	80	71	69	73	59	64	88	66	93	110	120	69	100
13C6-gamma HCH (225)	81	80	81	87	80	69	84	75	79	78	92	88	91
13C12-p,p'-DDE (330)	62	56	52	64	57	79	80	45	63	110	110	46	100
13C12-p,p'-DDT (247)	51	46	48	57	46	59	59	46	52	89	86	45	87
13C8-Mirex (277)	81	79	78	90	82	72	76	80	84	76	81	86	81
13C12-PCB 101 (338)	81	80	80	94	75	74	77	70	80	95	100	68	94
13C12-PCB 180 (406)	71	69	63	81	62	78	66	56	93	85	87	56	78
13C12-PCB 209 (512)	78	71	68	63	58	76	68	79	80	65	100	110	100

Values expressed as ng/g dry weight.
Values below detection limits shown as negative
Lab duplicates denoted by a "d" following the sample ID.
Values are recovery corrected.

Appendix 1

Chlorophenolics in Bed Sediment (Fall 1996)

Sample ID	96LCH1	96LCH4	96LCH4c	96LCH5	96LCH6	96MAN1	96MAN2	96MAN3	96MAN4	96NAR1	96NAR2	96NAR3	96NAR3c	96NAR4
4-CHLOROPHENOL	-0.33	-0.31	-0.3	-0.36	-0.26	-0.38	-0.13	-0.13	-0.13	-0.11	-0.34	-0.14	-0.14	-0.29
2,6-DICHLOROPHENOL	-0.21	-0.2	-0.2	-0.24	-0.19	-0.25	-0.14	-0.19	-0.19	-0.13	-0.34	-0.16	-0.13	-0.27
2,4/2,5-DICHLOROPHENOL	-0.5	-0.5	-0.55	-0.5	-0.25	-0.3	0.27	0.52	0.34	-0.15	0.56	-0.13	-0.23	-0.28
3,5-DICHLOROPHENOL	-0.24	-0.22	-0.23	-0.26	-0.21	-0.28	-0.16	-0.21	-0.22	-0.13	-0.37	-0.18	-0.14	-0.3
2,3-DICHLOROPHENOL	-0.24	-0.23	-0.23	-0.27	-0.22	-0.29	-0.16	-0.21	-0.22	-0.13	-0.37	-0.18	-0.14	-0.3
3,4-DICHLOROPHENOL	-0.21	-0.2	-0.2	-0.23	-0.19	-0.25	-0.13	-0.18	-0.18	-0.1	-0.3	-0.15	-0.12	-0.26
6-CHLOROGUAIACOL	-0.12	-0.1	-0.1	-0.1	-0.08	-0.12	-0.09	-0.1	-0.08	-0.08	-0.24	-0.09	-0.06	-0.14
4-CHLOROGUAIACOL	-0.14	-0.12	-0.12	-0.12	-0.09	-0.14	0.25	0.22	0.18	0.25	0.34	-0.11	-0.07	0.5
5-CHLOROGUAIACOL	-0.14	-0.12	-0.13	-0.12	-0.1	-0.15	-0.11	0.31	0.17	0.38	0.82	-0.19	-0.08	0.41
2,4,6-TRICHLOROPHENOL	-0.21	-0.2	-0.21	-0.24	-0.17	-0.23	-0.13	-0.16	-0.2	0.42	-0.45	-0.15	-0.11	-0.23
2,3,6-TRICHLOROPHENOL	-0.26	-0.25	-0.26	-0.3	-0.21	-0.29	-0.16	-0.21	-0.26	0.48	-0.51	-0.19	-0.14	-0.29
2,3,5-TRICHLOROPHENOL	-0.29	-0.27	-0.28	-0.32	-0.23	-0.32	-0.18	-0.24	-0.29	-0.18	-0.53	-0.22	-0.16	-0.33
2,4,5-TRICHLOROPHENOL	-0.21	-0.2	-0.21	-0.24	-0.18	-0.23	-0.12	-0.16	-0.19	-0.14	-0.42	-0.14	-0.1	-0.22
2,3,4-TRICHLOROPHENOL	-0.24	-0.22	-0.24	-0.27	-0.21	-0.26	-0.14	-0.19	-0.23	-0.15	-0.45	-0.17	-0.12	-0.25
3,4,5-TRICHLOROPHENOL	-0.25	-0.23	-0.24	-0.28	-0.21	-0.27	-0.14	-0.19	-0.23	-0.16	-0.46	-0.17	-0.12	-0.26
3-CHLOROCATECHOL	-0.32	-0.29	-0.32	-0.36	-0.27	-0.34	-0.22	-0.23	-0.28	-0.23	-0.51	-0.2	-0.15	-0.38
4-CHLOROCATECHOL	-0.34	-0.31	-0.34	-0.38	-0.29	-0.36	-0.24	0.25	0.52	0.28	1.2	-0.22	-0.17	-0.35
4,6-DICHLOROGUAIACOL	-0.3	-0.25	-0.27	-0.31	-0.27	-0.32	-0.14	-0.19	-0.19	-0.13	-0.39	-0.16	-0.12	-0.25
3,4-DICHLOROGUAIACOL	-0.39	-0.33	-0.36	-0.4	-0.35	-0.42	-0.18	-0.24	-0.25	-0.18	-0.53	-0.2	-0.15	-0.32
4,5-DICHLOROGUAIACOL	-0.35	-0.29	-0.32	-0.36	-0.31	-0.37	0.3	0.46	0.57	0.57	0.76	-0.19	-0.19	1.8
3-CHLOROSYRINGOL	-0.15	-0.14	-0.14	-0.17	-0.13	-0.16	-0.11	-0.2	-0.12	-0.14	-0.32	-0.12	-0.08	-0.18
3,6-DICHLOROCATECHOL	-0.54	-0.33	-0.36	-0.35	-0.31	-0.63	-0.28	-0.49	-0.31	-0.31	-0.94	-0.21	-0.17	-0.5
3,5-DICHLOROCATECHOL	-0.52	-0.32	-0.34	-0.33	-0.3	-0.6	-0.28	-0.48	-0.31	0.42	-0.88	-0.21	-0.16	-0.49
3,4-DICHLOROCATECHOL	-0.54	-0.4	-0.36	-0.35	-0.31	-0.75	0.63	-0.51	0.56	0.81	1.5	-0.22	-0.22	0.94
4,5-DICHLOROCATECHOL	-0.56	-0.34	-0.37	-0.36	-0.32	-0.65	1.6	0.58	0.62	2.1	2.5	-0.22	-0.21	1.7
2,3,5,6-TETRACHLOROPHENOL	-0.44	-0.37	-0.4	-0.46	-0.4	-0.47	-0.29	-0.45	-0.47	-0.27	-0.85	-0.36	-0.26	-0.49
2,3,4,6-TETRACHLOROPHENOL	-0.33	-0.28	-0.3	-0.34	-0.3	-0.35	-0.21	-0.33	-0.34	-0.2	-0.64	-0.27	-0.19	-0.36
2,3,4,5-TETRACHLOROPHENOL	-0.33	-0.28	-0.3	-0.34	-0.3	-0.35	-0.22	-0.34	-0.35	-0.18	-0.56	-0.27	-0.2	-0.37
5-CHLOROVANILLIN	-1.2	-0.81	-0.86	-0.98	-1.1	-1	-0.52	-0.95	-0.69	-0.7	-1.6	-0.6	-0.44	-0.86
6-CHLOROVANILLIN	-1.2	-0.81	-0.86	-0.98	-1.1	-1	1.6	1.3	1.8	1.5	2.6	-0.64	-0.74	3.6
3,5-DICHLOROSYRINGOL	-0.28	-0.24	-0.26	-0.29	-0.25	-0.3	-0.25	-0.36	-0.35	-0.39	-0.49	-0.32	-0.19	-0.46

Values expressed as ng/g dry weight.
Values below detection limits shown as negative

Appendix 1

Lab duplicates denoted by a "d" following the sample ID.

Chlorophenolics in Bed Sediment (Fall 1996)

Sample ID	96LCH1	96LCH4	96LCH4c	96LCH5	96LCH6	96MAN1	96MAN2	96MAN3	96MAN4	96NAR1	96NAR2	96NAR3	96NAR3c	96NAR4
3,4,6-TRICHLOROGUAIACOL	-0.27	-0.2	-0.2	-0.22	-0.19	-0.26	-0.2	-0.3	-0.34	-0.31	-0.79	-0.29	-0.19	-0.31
3,4,5-TRICHLOROGUAIACOL	-0.29	-0.21	-0.21	-0.23	-0.2	-0.27	-0.22	-0.33	-0.37	-0.29	-0.74	-0.32	-0.21	-0.67
4,5,6-TRICHLOROGUAIACOL	-0.18	-0.14	-0.14	-0.15	-0.13	-0.17	-0.15	-0.22	-0.25	0.77	-0.51	-0.22	-0.14	-0.23
3,4,6-TRICHLOROCATECHOL	-0.62	-0.41	-0.43	-0.45	-0.41	-0.84	-0.45	-0.49	-0.59	-0.41	-1.2	-0.39	-0.26	-0.6
3,4,5-TRICHLOROCATECHOL	-0.69	-0.46	-0.48	-0.5	-0.45	-0.93	1.2	0.94	1.1	1.1	2	-0.5	-0.3	2.2
5,6-DICHLOROVANILLIN	-0.43	-0.38	-0.38	-0.42	-0.35	-0.48	-0.23	-0.5	-0.58	-0.19	-0.47	-0.43	-0.23	-0.52
PENTACHLOROPHENOL	-0.36	-0.31	-0.31	-0.35	-0.29	-0.4	-0.23	-0.35	-0.36	-0.34	1	0.49	0.45	-0.45
2-CHLOROSYRINGALDEHYDE	-0.27	-0.24	-0.24	-0.26	-0.22	-0.3	-0.22	-0.27	-0.35	-0.11	-0.32	-0.26	-0.18	-0.42
3,4,5,6-TETRACHLOROGUAIACOL	-0.28	-0.25	-0.25	-0.27	-0.23	-0.3	-0.28	-0.38	-0.4	-0.2	-0.79	-0.26	-0.17	-0.57
3,4,5-TRICHLOROSYRINGOL	-0.33	-0.29	-0.29	-0.32	-0.27	-0.35	-0.26	-0.4	-0.49	-0.24	-0.72	-0.35	-0.23	-0.65
3,4,5,6-TETRACHLOROCATECHOL	-0.65	-0.93	-1	-0.53	-0.56	-2.2	2.1	0.81	1.1	1.9	-4.1	-0.75	-0.88	2.4
2,6-DICHLOROSYRINGALDEHYDE	-0.2	-0.18	-0.18	-0.2	-0.16	-0.22	-0.18	-0.26	-0.38	-0.19	-0.25	-0.18	-0.19	-0.34
% Surrogate Recovery														
4-CHLOROPHENOL-13C	78	79	81	74	96	69	63	68	65	92	77	72	54	72
2,4-DICHLOROPHENOL-13C	91	88	88	82	106	77	67	72	70	88	79	78	65	79
4-CHLOROGUAIACOL-13C	69	89	84	82	101	76	57	63	64	69	65	72	60	71
2,4,6-TRICHLOROPHENOL-13C	82	80	77	73	97	74	67	71	70	90	79	76	66	77
2,4,5-TRICHLOROPHENOL-13C	85	86	81	76	96	79	74	78	79	90	79	85	76	85
5-CHLOROVANILLIN-13C	52	75	70	67	57	52	66	67	75	45	51	76	66	86
2,3,4,5-TETRACHLOROPHENOL-13	57	68	62	57	62	56	45	63	65	65	56	62	54	71
4,5-DICHLOROCATECHOL-13C	42	62	60	58	62	35	24	34	52	43	37	61	53	73
4,5,6-TRICHLOROGUAIACOL-13C	56	75	75	70	76	61	45	61	60	58	52	59	54	73
PENTACHLOROPHENOL-13C	64	71	72	66	76	59	49	66	61	66	61	61	54	76
3,4,5,6-TETRACHLOROGUAIACOL-	62	69	68	65	73	61	49	67	63	67	62	60	51	72
3,4,5,6-TETRACHLOROCATECHOL-	16	18	17	32	29	8	3	14	40	14	8	16	10	32

Values expressed as ng/g dry weight.
Values below detection limits shown as negative
Lab duplicates denoted by a "d" following the sample ID.