BIOLOGICAL EFFECTS OF ESTROGENIC AGENTS: INDUCTION OF DNA DAMAGE;
RECEPTOR ACTIVATION AND JOINT ACTION

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ABSTRACT

Biological Effects of Estrogenic Agents: Induction of DNA Damage; Receptor Activation and Joint Action

Breast and testicular cancer incidence continues to rise as does the number of reports of abnormal sexual development in Man and wildlife. A widely held belief is that estradiol and environmental contaminants capable of mimicking the hormones' actions may be implicated when the mechanisms are finally elucidated.

17ß-Estradiol (E₂) has been proposed as a carcinogen capable of tumour initiation by mutation. The ability of estradiol to induce DNA damage by undergoing metabolic activation to catechol estrogens in breast cancer cells was investigated. The hormone, at concentrations as low as 10 nM, caused DNA fragmentation in the Comet assay. The presence of 7,8-dihydro-8-oxo-guanine and oxidised purines was inferred from the observation that formamidopyrimidine glycosylase digestion of treated DNA led to increases in DNA damage. This suggests free-radical attack by redox cycling of catechol estrogen quinones. These quinones are believed to be involved in cell progression from the normal to the malignant state.

The importance of assessing xenoestrogen (XE) mixtures is becoming more widely appreciated. Our goal was to test the hypothesis that, although XEs are considerably less potent than steroidal estrogens, they can significantly impact on the effects of the hormones. We carried out extensive concentration-response analyses of E₂ and XEs in a yeast reporter-gene assay with the α-human estrogen receptor. Two pharmacological models, concentration addition and independent action, were used to calculate effects for multiple-component mixtures. Observations confirmed decisively the additivity predictions and the impact of the XEs was significant. In a mixture with eleven XEs, mixed at ratios proportional to their EC₀₁ values, before being combined with E₂ at a mixture ratio of 1:25000 (E₂:XE-pool), the XEs, whose concentrations were below individual effect-thresholds, resulted in an 80% greater response than the hormone alone. By ignoring additive combination effects one might wrongly conclude that each XE presents no risk.
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GLOSSARY

Concentration addition (CA)
A reference model for computing combination effects on the basis of the dose-response relationships of individual mixture components. Its origins can be traced to the early work of Frei [1913], and Loewe and Muischnek [1926]. CA assumes that chemicals act in a similar manner and states that effects can be produced by replacing one compound totally or in part with other constituents. Each individual component is thought to contribute to the overall mixture effect by acting in proportion to its concentration, even below threshold concentrations. See also effect summation and independent action.

Effect summation (ES)
Method for computing combination effects of mixtures containing compounds with linear concentration-response relationships, by simply summing the effects of each individual component. It produces unreliable results when dealing with mixtures of agents showing sigmoidal curves with differing maximal effects and slopes. See also concentration addition and independent action.

Independent action (IA)
A reference model for computing combination effects on the basis of the dose-response relationships of individual mixture components. Developed by Bliss [1939], it assumes that compounds act on different subsystems in organisms. When present at sub-threshold doses, mixture components will not contribute to mixture effects. See also concentration addition and effect summation.

Mutation
Generally, any base change that is not repaired and is transmitted to the cell’s offspring, whether it results in a pathological consequence or not. Mutation may be spontaneous (the result of accidents in the replication of genetic material), or induced by external factors (e.g., electromagnetic radiation and certain chemicals).

No Observed Effect Concentration (NOEC)
A term used in risk assessment. NOEC values are usually derived from experimental data by applying statistical hypothesis testing procedures and only denote “the highest test concentration
at and below which the effect parameter does not depart significantly (in a statistical sense) from the effect parameter observed in the control" [Van der Hoeven, 1997].

Phytoestrogen
Any estrogenic compound synthesised in plants. Examples include diadzein and genistein from soy.

Reactive Oxygen Species (ROS)
Species formed by oxygen readily accepting electrons from other molecules. Many types of reactive oxygen species exist in the cellular environment, the most important being hydrogen peroxide (H₂O₂), the superoxide anion (O₂⁻), and the highly reactive hydroxyl radical (OH⁺). ROS are known to damage cellular macromolecules including DNA.

Redox cycling
The alternate acceptance and donation of an electron by an agent (in our case, a catechol estrogen quinone), leading to the production of ROS.

Tail moment
A measure of DNA fragmentation, as determined using the Comet assay. It is defined as the product of tail length and % DNA in the tail, the result has the units microns (µm). Two types are commonly quoted, i.e. Olive tail moment and Tail extent moment, the difference being the definition of tail length.

Xenoestrogen (XE)
Literally "foreign estrogen". Any chemical, not naturally synthesised in the body, capable of mimicking the endogenous steroidal estrogens.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>8-OHgua</td>
<td>7,8-dihydro-8-oxo-guanine</td>
</tr>
<tr>
<td>α-MEM</td>
<td>α-modified Eagle’s medium</td>
</tr>
<tr>
<td>β-gal</td>
<td>β-galactosidase</td>
</tr>
<tr>
<td>ALS</td>
<td>alkali-labile site</td>
</tr>
<tr>
<td>BPA</td>
<td>bisphenol A</td>
</tr>
<tr>
<td>CA</td>
<td>concentration addition</td>
</tr>
<tr>
<td>CDHus</td>
<td>charcoal dextran stripped human serum</td>
</tr>
<tr>
<td>CI</td>
<td>confidence interval</td>
</tr>
<tr>
<td>CPRG</td>
<td>chlorophenol red-β-galactopyranoside</td>
</tr>
<tr>
<td>CYP1A1</td>
<td>cytochrome P450 1A1</td>
</tr>
<tr>
<td>CYP1B1</td>
<td>cytochrome P450 1B1</td>
</tr>
<tr>
<td>DDT</td>
<td>dichlorodiphenyl trichloroethane</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DSB</td>
<td>DNA double strand break</td>
</tr>
<tr>
<td>E₁</td>
<td>estrone</td>
</tr>
<tr>
<td>E₂</td>
<td>17β-estradiol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diammine tetraacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>estrogen receptor</td>
</tr>
<tr>
<td>ES</td>
<td>effect summation</td>
</tr>
<tr>
<td>EtOH</td>
<td>ethanol</td>
</tr>
<tr>
<td>FPG</td>
<td>formamidopyrimidine glycosylase</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
</tr>
<tr>
<td>IA</td>
<td>independent action</td>
</tr>
<tr>
<td>NMP/LMP</td>
<td>normal/low melting point (agarose)</td>
</tr>
<tr>
<td>OTM</td>
<td>Olive tail moment</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SHBG</td>
<td>sex hormone-binding globulin</td>
</tr>
<tr>
<td>SCGE</td>
<td>single cell gel electrophoresis (Comet) assay</td>
</tr>
<tr>
<td>SSB</td>
<td>DNA single strand break</td>
</tr>
<tr>
<td>UDS</td>
<td>unscheduled DNA synthesis</td>
</tr>
<tr>
<td>UHQ</td>
<td>ultra high quality water</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>XE</td>
<td>xenoestrogen</td>
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<tr>
<td>YES</td>
<td>yeast estrogen screen</td>
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</table>
CHAPTER 1 – INTRODUCTION

Cellular homeostasis throughout the body is regulated by the immune, nervous and endocrine systems, enabling the body’s internal environment to be altered in response to changes in the external environment.

The endocrine (endo = within; crin = to secrete) system is a group of ductless glands that regulate body processes by their secretion of chemical substances called hormones (hormone = set in motion), which are carried to specific target organs and tissues by the bloodstream. The endocrine system’s three parts; glands, hormones and target cells, relay information and instructions throughout the body. Sometimes the whole process works within seconds, say, in response to fear. At other times the effects occur more slowly, for instance telling body parts when and how much to grow and developing characteristics that distinguish male from female. It is exquisitely self-regulating so that any disruption of the normal internal environment is resisted by powerful countermeasures. When this resistance is overcome, sickness ensues.

1.1 Steroid hormones

Most commonly, hormones are categorised into four structural groups, with members of each group having many properties in common, they are; proteins and peptides, fatty acid derivatives, amino acid derivatives and steroids. This thesis is concerned with the steroid hormones, in particular estradiol and exogenous compounds capable of acting in a way similar to the hormone.

Derived from cholesterol, steroid hormones have effects at all levels of biological organisation, and elicit their numerous effects in mammalian cells and tissues by binding to receptors that function directly as ligand-activated transcription factors. The receptors belong to the superfamily of, largely, intracellular receptors which includes the retinoid, thyroid hormone and vitamin D receptors as well as many other orphan receptors [Mangelsdorf et al., 1995].

The cognate ligands for steroid receptors tend to be lipophilic and membrane-permeable molecules. According to the textbook view on steroid receptor activation, the inactive complex of aporeceptor and molecular chaperones dissociates upon binding of
steroid hormone to its receptor. Receptor-ligand complexes then dimerise and bind to specific response elements on DNA, which in association with several transcriptional coactivators, results in the stimulation of the transcription of genes linked to the response elements. This widely accepted view of activation as being strictly dependent on the interaction between hormone and receptor is not, however, the whole story. Evidence is emerging that alternative pathways of steroid receptor activation exist and it appears that several steroid receptors can be activated in the absence of cognate hormones [reviewed in Cenni and Picard, 1999].

As previously mentioned, the effects of the various steroid hormones are numerous and diverse. A discussion of these effects beyond what has preceded is superfluous to this particular story. It is at this point that the description will concentrate on the role and molecular mechanisms of action of estradiol.

1.1.1 Estrogen synthesis

Estrogens are synthesised in the ovary and to a minor degree in the adrenal cortex in response to follicle stimulating hormone (FSH). Production of estrogens is also seen in large amounts in the placenta and small amounts in the testis. Steroid precursors can also be converted into estrogens in the liver, muscle, fat and hair follicles.

In males, estradiol is primarily produced by the Leydig cells and, to a lesser degree, adipocytes. It has been suggested, however, that it is unlikely that the unchanging levels of estradiol in the blood can regulate functions at the numerous sites in the male where estrogen receptors are expressed [Sharpe, 1998]. Instead, the findings are beginning to point to local conversion of testosterone to estradiol by the enzyme aromatase, which has been detected in parts of the brain [Hutchison et al., 1997], adipose tissue [Simpson et al., 1994], bone [Jakob et al., 1997] and the heart [Grohe et al., 1998].

The biosynthesis pathway begins with cholesterol and the most important reaction is the aromatization of the A ring of androstenedione or testosterone (Figure 1.1). The main estrogen synthesised is 17β-estradiol (E₂), whilst smaller amounts of estrone are produced, with an equilibrium existing at a ratio of between 1:2 and 1:4 estrone to estradiol. A third weak estrogen formed from estrone is estriol.
1.1.2 Actions of estrogens

Plasma concentrations of estrogens in women vary depending on the phase of the menstrual cycle or if they have reached the menopause (Table 1.1). The majority (>70%) of the circulating estrogens are loosely bound to the plasma protein sex hormone-binding globulin (SHBG) and approximately 25% is bound to plasma albumin. The unbound or free fraction is considered active [Toniolo et al., 1995].

| Table 1.1 Normal ranges of total serum estrogens in women and men [Taken from Wilson et al., 1998]. |
|---|---|---|---|
| **Sex** | **Phase** | **Serum levels (pmol/L)** |
| Women | Follicular | 75-220 |
| | Mid follicular | 300-1500 |
| | Luteal | 200-1100 |
| | Post menopausal | < 200 |
| Men | - | < 175 |

*1 pmol/L is equivalent to 0.27 pg/mL

The main actions of estrogens are in the development of female secondary sexual characteristics and maintenance of the reproductive tract. They also play a crucial role in the menstrual cycle. Apart from these effects, estrogens induce a degree of salt and fluid retention by stimulating angiotensinogen production in the liver, as well
as lowering the levels of circulating cholesterol. Growth and development of the ductile system in the breast is also stimulated by estrogens, facilitating lactation. During pregnancy, estrogens are important stimulators of myometrial growth and they sensitise the myometrium to the action of oxytocin before parturition.

More recently, rapid estrogen effects, not induced via the classical estrogen receptor-transcriptional activation of estrogen-responsive genes, have been discovered. In isolated pulmonary endothelial cells, 17β-estradiol-induced rapid activation of endothelial nitric oxide synthase has been described, explaining the ability of estrogens to induce acute dilation of blood vessels [Chen et al., 1999]. Singer et al. [1999], reported a similar rapid non-genomic action for estrogen, but in primary cortical neurones where estrogen’s rapid activation of the MAP kinase signalling pathway resulted in neuroprotection after glutamate excitotoxicity.

Continued research in this field has expanded the function of steroid hormone receptors beyond the confines of sexual differentiation and reproductive neuroendocrine function and may eventually yield new therapeutic strategies for numerous defects.

1.1.3 Estrogen receptors

It was the work of Jensen and Jacobson that eventually led to the discovery of the estrogen receptor (ER). They revealed the temporary accumulation of estradiol in the rat uterus for a few hours prior to any signs of proliferative activity being manifested [Jensen and Jacobson, 1962]. The protein responsible for the brief retention of estradiol inside target cells was the estrogen receptor, the human estrogen receptor (hER) was eventually cloned in 1986 [Green et al., 1986; Greene et al., 1986], and is now known as ERα.

For a long time, the effects of estrogen were believed to be mediated by this receptor, however recently, a second receptor, ERβ, has been cloned [Mosselman et al., 1996; Enmark et al., 1997]. Following the discovery of the second isoform, tissue distributions for the two receptors were investigated [Kuiper et al., 1996]. hERβ is the predominant subtype in bone, blood vessels and in the brain, whereas its content in uterus and liver is low. The ER subtypes share approximately 55% identical amino acid residues in their ligand-binding domains (LBDs), but in spite of this conservation an ERα-specific ligand has been demonstrated to exhibit a more than 100-fold difference in binding affinity [Sun et al., 1999].
Estradiol’s actions are primarily executed by its binding to these nuclear receptors, i.e. ERα and ERβ, which are ligand-inducible transcription factors [Tsai and O'Malley, 1994; Beato et al., 1995]. They modulate transcription of genes by binding as estrogen-estrogen receptor complexes to specific DNA sequences (estrogen responsive elements) in target promoters (Figure 1.2).

![Figure 1.2 - Mechanism of estrogen receptor action.](image)

Estrogen receptors (ER) constantly shuttle between the cell nucleus and the cytoplasm, but under steady-state conditions they are predominantly nuclear [King and Green, 1984]. Upon binding of hormone (L), the inactive receptor, complexed with heat shock proteins, e.g. hsp90, hsp70 and p59, undergoes a conformational change. This allows receptor dimerisation and high-affinity DNA binding to estrogen responsive elements (EREs). Transcription of genes by RNA polymerase (pol) II is initiated downstream of a TATA box that binds TFIIID, a complex comprising a TATA box-binding protein (TBP) and TBP-associated factors (TAFs). The TATA box may also bind a number of other basal transcription factors, including TFIIA (A), TFIIB (B), TFIIE (E), TFIIF (F), and TFIIH (H).

The myriad of estrogenic effects, previously described, that occur rapidly within seconds or minutes, are clearly not modulated via the classical pathway detailed in Figure 1.2. These responses do not require RNA or protein synthesis and are considered to be mediated by estrogen binding to the plasma membrane [Lieberherr et al., 1993; Aronica et al., 1994; Watson and Gametchu, 1999]. However, although evidence from numerous laboratories supports the existence of such a receptor [Pietras and Szago, 1977; Le Mellay et al., 1997], it is yet to be isolated and structurally or functionally characterised. Recently
though, Nadal et al. [2000], showed that xenoestrogens and 17β-estradiol bind to a common membrane binding site that is shared by dopamine, adrenaline and noradrenaline, and has the pharmacological profile of the γ-adrenergic receptor.

1.1.4 Control of steroid receptor concentration

The observation that estrogen target cells are capable of maintaining a constitutive level of ER, even in the castrated animal, does not indicate a complete lack of influence of gonadal hormones on ER levels. ER levels are influenced by endogenous and exogenous estrogens. The injection of E₂ causes a rapid depletion of unbound receptors, which bind tightly in the nucleus as estrogen-receptor complexes. This is followed by a period during which the unbound fraction of ER is replenished by at least two processes; the reactivation or reutilisation of bound receptor and de novo ER synthesis. In tissues such as the uterus, vagina and mammary glands, that grow either by hyperplasia or by hypertrophy in response to estrogen stimulation, both responses may be involved to maintain a constant ER number per cell following division.

Estrogens also have an impact upon the levels of progesterone receptors. The uterus is relatively insensitive to progesterone unless first exposed to estrogen. The priming by estrogen stimulates the uterus to synthesise progesterone receptors, thereby enhancing the uterine response to progesterone. The effect is not purely quantitative, changes in the form of progesterone receptor have also been noted [Junne et al., 1976]. It is of interest that progesterone acts to decrease the concentration of unoccupied ER [Hsueh et al., 1975], which correlates with a decreased ability of estrogens to stimulate uterine growth. In this way, progesterone may suppress the synthesis of its own receptor by desensitising the uterus to estrogen.

The levels of ER in a hormone responsive tissue will clearly be affected by levels of unbound estrogens, both endogenous and exogenous, and also to some degree by the levels of circulating progestins.
1.1.5 Estrogens: villains or saviours?

Estrogens play many integral physiological roles in the body, as described earlier, being principally necessary for the development and maintenance of healthy reproductive systems in both Man and wildlife. Links are also becoming apparent between estrogen exposure and other beneficial health effects. For instance, estrogens appear to offer protection from cardiovascular problems, osteoporosis and even possibly Alzheimer’s disease [Slootweg et al., 1997]. Sadly, the final account appears destined not to end with estrogens simply being beneficial.

The stimulatory actions in hormone responsive tissues such as the breast and uterus now link estrogens with the development of some cancers. Experimental studies on animals, carried out by Bittner [1948], on estrogens and mammary cancer led to the concept that hormones can increase the incidence of neoplasia. The weight of experimental and epidemiological data supporting the hypothesis that estrogens increase the risk of developing certain forms of cancer is substantial. However, the mechanisms are still to be elucidated. Recently, the Scientific Committee on Veterinary matters relating to Public Health (SCVPH) delivered an opinion on the potential risks to human health from hormone residues in bovine meat and meat products †. The Committee concluded that for the six hormones under review (17β-estradiol, testosterone, progesterone, melengestrol acetate, trenbolone and zeranol), endocrine, developmental, immunological, neurobiological, immunotoxic, genotoxic and carcinogenic effects could be envisaged. It also concluded that there is a substantial body of recent evidence suggesting that 17β-estradiol has to be considered as a complete carcinogen.

The contradictory roles of estrogens are not restricted solely to the endogenous hormones. Drugs developed to either block estrogens’ bad effects or mimic the good ones also demonstrate two sides. Tamoxifen, commonly prescribed to treat or prevent breast cancer, is very effective at blocking the cancer-promoting effects of estrogen in breast tissue, but acts as an estrogen in the uterus, where it increases the risk of uterine cancer.

Our understanding of the many aspects of estrogen action is far from complete, as can be readily seen above from drug development, and it fails to get any simpler. As

† The full report can be accessed via the European Union website, at the following internet address: http://europa.eu.int/comm/dg24/health/sc/scv/index/en.html
the number of chemicals being produced worldwide continues to rise unabated, large numbers of these compounds are being identified as endocrine-active chemicals or endocrine disrupters. Many are being reported as "estrogenic," i.e. interacting with the ER or mimicking the actions of 17β-estradiol. The first of these so-called xenoestrogens to be identified were two pesticides, technical grade dichlorodiphenyl trichloroethane (DDT) and methoxychlor [Tullner, 1961; Bittman et al., 1968; Welch et al., 1969]. It has also been reported that plants produce chemicals that are estrogenic, known as phytoestrogens, these natural compounds are commonly found in the diet [Makela et al., 1995].

The question arises as to whether xenoestrogens are beneficial [Adlercreutz, 1995] or whether a high degree of risk is associated with exposure to these chemicals.

1.2 Estrogens as a cause of detrimental health effects

Breast and testicular cancer incidence continues to rise, as do the number of reports of abnormal sexual development in Man and wildlife. The evidence continues to amass indicating a pivotal role for estrogens in these conditions.

One of the most important and dramatic effects of estrogens is a superpotent mitogenic action in hormone sensitive tissues such as the uterus [Quarmby and Korach, 1984], breast [Holland and Roy, 1995], ovary and prostate. Prolonged exposure of tissues to excessive mitogenic stimulation by endogenous or synthetic steroidal estrogens has long been considered an important etiological factor in the induction of estrogen-associated cancers [Nandi et al., 1995; Feigelson and Henderson, 1996; Li and Li, 1998]. Apart from the controversial proposal that breast cancer is linked to exposure to xenoestrogens, other less common cancers have been shown to be associated specifically with exposure to synthetic estrogens. Exposure in utero to diethylstilbestrol (DES) resulted in an increased incidence of cervicovaginal clear-cell carcinomas in young women and of testicular cancer in men [Henderson et al., 1988], while prolonged use of anabolic steroids and oral contraceptives is associated with an increased risk for developing hepatocellular adenomas and carcinomas [Palmer et al., 1989].

As previously mentioned, the most widely appreciated, and therefore investigated, effect of estrogens is increased cell proliferation [Preston-Martin et al., 1990]. More recently though, it has become clear that several estrogen metabolites can directly
interact with DNA or, via redox cycling processes that generate reactive radical species, cause oxidative DNA damage [Liehr and Roy, 1990]. Research continues based largely around the two hypotheses that estrogens are involved in the development of cancer by either stimulating cellular proliferation or causing DNA damage and thereby mutagenic change. As Davis and Bradlow [1995], eloquently described it: by impacting on interacting genetic and hormonal pathways critical for breast carcinogenesis:

Naturally occurring or synthetic xenohormones may affect the process of tumorigenic transformation at both genotoxic (initiational) or epigenetic (post-initiational) levels.

1.2.1 Epidemiology and prevalent hypotheses

In the early 1990s, the epidemiologic evidence did not suggest associations between endogenous estrogens measured in blood and urine and breast cancer risk [Bruning et al., 1992]. However, in the space of just two years, four studies [Helzlsouer et al., 1994; Toniolo et al., 1995; Berrino et al., 1996; Dorgan et al., 1996], provided considerable momentum to just that hypothesis, i.e. that cumulative lifetime exposure to estrogens is a major risk factor for the development of breast cancer.

The reason for the sharp contrast with previous data was attributed to the design of the studies [Toniolo et al., 1997]. The earlier studies tended to involve the identification of breast cancer cases among patients attending medical facilities for diagnosis or treatment. In this so-called case-control design, assessment of exposure to endogenous hormones is performed among the cases on biological specimens that are obtained at the time, or sometimes long after, breast cancer has become clinically manifest. This introduces uncertainty as to whether exposure and disease occur in the correct temporal sequence - one of the most fundamental prerequisites of epidemiological studies. It is Toniolo’s contention that it is not possible to reasonably assume that the presence of the disease at the clinical stage does not influence hormonal measurements which, in turn, are used to provide a reflection of hormonal levels during an appropriate time in the natural history of the disease. In agreement with Toniolo, it has been shown that breast tumours do in fact take up estradiol via a high affinity uptake mechanism, and that although the level of estradiol in serum may be low the concentration in the tumour may be an order of magnitude higher [Masamura et al., 1997].
A more appropriate method, Toniolo argues, is conduction of case-control studies nested in a prospective cohort, as was done in the studies published between 1994 and 1996. Here, the assessment of exposure is performed on samples collected from all or most of the cohort prior to clinical disease onset. Biochemical markers are then measured on samples from women who later develop breast cancer and controls drawn from among the non-disease members. This approach clearly allows any temporal sequence to be established. Based upon the available data in the early 1990s:

The current chapter of the epidemiology of breast cancer concerned with the role of reproductive hormones could have ended ... with the conclusion that, contrary to expectations, endogenous estrogens measured in blood and urine do not reflect breast cancer risk.

Toniolo, 1997

Why did the epidemiologists take such a long time to recognise the key role of endogenous hormones? The results of the epidemiological research of the late 1960s and 1970s were conflicting, with the majority showing no association between urinary and circulating estrogens and breast cancer [England et al., 1974; Reed et al., 1983], whilst a number observed a modest positive relationship [Morreal et al., 1979; Bruning et al., 1985]. Siiteri and colleagues [1981], then reported that only the free and albumin-bound fractions of estradiol, rather than that bound to sex hormone-binding globulin, are relevant. This was confirmed by the large proportion of studies to follow [Mahon et al., 1983; Moore et al., 1982], but their overall impact was reduced by the knowledge that this association was not of sufficient strength to explain much of the breast cancer epidemiology. It was at this point that groups began to look at this problem with prospective cohort studies [Bulbrook, et al., 1986; Wysowski et al., 1987], however these studies suffered from small sample sizes and short follow-up periods and as a result findings were inconsistent. It was not until properly designed prospective cohort studies, with sufficiently large cohorts, that a positive association between increasing risk of breast cancer in postmenopausal women and increasing excretion of estradiol and total estrogens was demonstrated.

Breast cancer is now the most common cancer in women throughout the world [WHO, 1997]. Approximately 20%, some 600 000 cases, of the cancers diagnosed worldwide each year are cancers of the breast, and despite major advances in treatment the mortality rate has not decreased. There has been a gradual and persistent rise in breast cancer incidence since 1940 [Harris et al., 1992], on average 1% per year since 1973 [Kosary et al., 1994]. However, this increase cannot be accounted for solely by changes in
known risk factors or by the increase in mammographic screening and other diagnostic techniques.

It is well established that a family history of breast or ovarian cancer constitutes a risk factor for breast cancer. This has largely been attributed to the inheritance of germ cell line mutations in genes such as BRCA1, BRCA2, p53 or pRB. Although such mutations account for as many as 90% of cases in some families, less than 10% of all breast cancer cases are in women who have inherited these mutations [Castilla et al., 1994].

Among non-demographic factors, the most consistently documented risk factors for breast cancer are early age at menarche, late age at menopause, late age of first full-term pregnancy and weight. Early age at menarche has been demonstrated as a risk factor in most case-control studies [MacMahon et al., 1973]. In general, a 20% decrease in breast cancer risk results from each year that menarche is delayed. Women with early menarche and rapid establishment of regular cycles have an almost four-fold increased risk of breast cancer [Henderson et al., 1981]. Further supportive evidence for the concept that cumulative estrogen exposure is a major determinant of breast cancer came from the international studies of MacMahon et al. [1982].

MacMahon et al. [1970], observed that there was a decreased risk of breast cancer with increased parity. They found that nulliparous women, whether single or married, were approximately 1.4 times more at risk of developing breast cancer than parous women. The main finding of this study was that the protective effect of parity was actually due to a protective effect of early age at first birth.

There is a strong correlation between weight and breast cancer, however the relationship is critically dependent on age. For women aged under 50 the connection is weak if present at all, but by age 60 an increment of 10 kg in weight results in approximately an 80% increase in risk [Tretli, 1989]. A possible explanation for this could be the fact that the primary source of estrogens in post-menopausal women is from the conversion of androstenedione to estrone in adipose tissue, thus post-menopausal obesity increases risk of breast cancer through increased production of estrogens. Obesity is also associated with decreased sex hormone-binding globulin (SHBG) production. The consequence of this being an increased proportion of free and albumin-bound estradiol which are understood to be the biologically active estrogens [Bernstein and Ross, 1993]. As with breast cancer, the importance of weight in determining the risk of endometrial cancer is well documented, principally for the same reasons [MacDonald et al., 1978].
Mechanistic studies of carcinogenesis indicate an important role for endogenous oxidative damage to DNA that is balanced by elaborate defence and repair processes. The rate of cellular proliferation has been shown to be key, as this is a determining factor of the probability the DNA lesions are converted to mutations [Liehr et al., 1988]. It is well established that estrogens have a stimulatory effect on cellular proliferation, as do many xenoestrogens [Soto et al., 1995], and it now appears that estrogen metabolism may also induce DNA damage under certain conditions [Nutter et al., 1991; Han and Liehr, 1994; 1994b; 1994c; Li et al., 1994].

As indicated earlier, the rise in breast cancer incidence is not completely explained in terms of changes in known risk factors. As a result, the proposal that exposure to environmental chemicals capable of mimicking the effects of 17β-estradiol adds to the body's estrogenic "load" and thus increases the associated risks is gaining support. At the same time as this proposal was being formulated others were suggesting a role for xenoestrogens that might explain the detrimental health effects seen in male reproductive function [Sharpe and Skakkebaek, 1993].

For instance, in a comprehensive meta-analysis of 61 studies that had investigated semen quality, Carlsen et al. [1992], concluded that mean sperm counts had almost halved in the 50 years the studies covered. Likewise, groups reported increased incidence of cryptorchidism and hypospadias (maldescent testis and urethral abnormalities, respectively) [Paulozzi, 1999] over similar time periods. Man is not the only species affected, many animal species were also seen to be affected by similar disorders [Colborn et al., 1993].

For the mammalian foetus to develop into a male, there is the requirement for hormone (particularly androgens and anti-mullerian hormone) activation of specific pathways. Interference with this activation or in hormone production may lead to partial or complete prevention of masculinisation. All aspects of masculinisation (except testis formation) are dependent on normal testis function and the consequent production of adequate amounts of hormones. In effect, disturbance of testicular development will most likely have serious repercussions on the development health of the individual.

If either the Sertoli cells of Leydig cells malfunction, testicular dysgenesis will result. The downstream consequences of this could include incomplete masculinisation (hypospadias or cryptorchidism) and even testicular cancer [Toppari et al., 1996], arising from pre-malignant gonocytes [Skakkebaek et al., 1987]. Sharpe [1998], describes the unperturbed male as involved in a delicate balancing act between androgens and
estrogens. In apparent agreement with this description are the recent findings that seem to link the perturbed state with the question of why gonocytes sometimes maldevelop [reviewed in Sharpe, 2001]. Basically, the hypothesis gaining support suggests that lowered androgen production, resulting from abnormal Sertoli cell of Leydig cell function, leads to altered estrogen production and it is these that act on gonocytes via the ERβ [Saunders et al., 1998], and lead to aberrant cell proliferation. Clearly, such effects may not be exclusive to endogenous hormones, evidence suggests that anti-androgens (and to some degree, xenoestrogens) may also cause male reproductive disorders when exposure is during critical developmental windows [Toppari et al., 1996; Gray et al., 1999; Foster et al., 2000].

1.2.2 Xenoestrogens and observations in wildlife

The most notable findings, for the weight of evidence they provided supporting this hypothesis, were in the alligator population of Lake Apopka, Florida, and the western gulls off the coast of California. In Lake Apopka, a drop in the alligator population was observed between 1980 and 1987 [Guilette Jr. et al., 1994]. The juvenile alligator population exhibited reproductive anomalies, including small phallus size, reduced hatching success and poor survivorship. The effects were attributed to the high levels of halogenated pesticides {1,1,1-trichloro-2,2-bischlorophenylethane (DDT), 1,2-dibromo-3-chloropropane (DBCP) and ethylene bromide (EDB)} to which the population had been exposed since 1980, when there had been an extensive spill from the Tower Chemical Company facility [Semenza et al., 1997]. When Vonier et al. [1996] showed that these chemicals interact with the estrogen and progesterone receptors the link seemed undeniable.

In California, the western gull population was also seen to decrease significantly. Poor breeding success was due to fewer adult males, of which some demonstrated feminisation [Fry and Toone, 1981]. The researchers noted similar changes in other avian species in the area and associated these profound changes to the high usage of DDT in the late 1960s and early 1970s.

Probably the best-studied species, however, is fish. Regardless of the source of use, substantial amounts of synthetic chemical compounds end up in the aquatic environment [reviewed in Arukwe and Goskoyr, 1998]. Whiting caught off the coast of The
Netherlands and off the Rhine River Estuary had high malformation rates, corresponding to the positive correlation between anaphase aberrations and the levels of organochlorines such as polychlorinated biphenyls (PCBs), DDT and DDE in their gonads and livers [Cameron et al., 1988]. Alkylphenolic compounds were thought to be responsible for the observations of Purdom and colleagues [1994], in male trout. They demonstrated that sewage effluent contained estrogenic chemicals that induced male trout to synthesise the egg yolk precursor vitellogenin. Further studies have shown that where estrogenicity can be shown to occur in rivers and estuaries of the United Kingdom, these waters are also recipients of sewage effluent [Harries et al., 1996; 1997; Lye et al., 1997]. Numerous other reports detailing reproductive effects of contaminants of the aquatic environment on marine organisms are reviewed by Arukwe and Goksyr [1998].

1.2.3 The final link?

There is increasing evidence to link the development of breast, testicular and ovarian cancer and other endocrine related disorders with high cumulative lifetime exposure to endogenous estrogens. However, there is still a lack of understanding concerning the mechanisms involved. The indication is that there is a prominent role for xenoestrogens in the increased incidence of these diseases, but epidemiological studies fail to show conclusive links between the detrimental health effects and environmentally prevalent levels of any single xenoestrogen [Safe, 1995].

The field remains at odds concerning whether any links exist between breast cancer incidence and xenoestrogen levels in human tissues. The most widely studied pesticide has been DDT, an insecticide first used during World War Two for control of lice and mosquitoes to combat typhus and malaria [Hayes, 1991]. The evidence that led to a detailed investigation of DDT and DDE included the previously mentioned wildlife observations such as reproductive defects and eggshell thinning in avian species [Fry and Toone, 1981; Fry, 1995], and the ability of technical DDT and o,p'-DDT to support the growth of estrogen-dependent breast tumours in rats. Its environmental persistence and ability to bio-concentrate in adipose tissue up the food chain were also seen as significant factors [Teschke et al., 1993]. The more recent epidemiological studies have tended to indicate no correlation between serum or adipose levels of DDT/DDE and risk of breast cancer [Krieger et al., 1994; Hunter et al., 1997; van't Veer et al., 1997], although earlier
studies had seen a positive correlation [Falck et al., 1992; Wolff et al., 1993]. These studies were carried out in the USA and Europe after the use of the insecticide had been banned. However, in countries with a more recent history of DDT usage, such as those in South America, studies have found an elevated risk of breast cancer in women with higher serum levels of DDE [Olaya-Contreras et al., 1998; Romieu et al., 2000]. A comprehensive review of the numerous epidemiological studies noted that the lack of consistency in findings could be due to any number of factors including, exposure to different forms of DDT, analytical methodologies, dietary factors, menopausal status, use of different control populations and estrogen receptor status [Snedeker, 2001]. The author concludes that these confounding factors need to be considered when carrying out future epidemiological studies in countries "such as India that have a long and continuing history of DDT use."

An impasse appears to have been reached. Although increasing numbers of environmentally relevant compounds are being identified as estrogenic [Soto et al., 1994, Routledge and Sumpter, 1996; Blair et al., 2000], the vast majority of these chemicals are considerably less potent than the steroidal estrogens and are usually present in human tissue at levels that induce insignificant effects. Their low potency in relation to 17β-estradiol is often used to argue that xenoestrogens in combination with steroidal estrogens will not produce effects distinguishable from those of the steroid [Safe, 1995b]. This fuelled the belief that synergisms between xenoestrogens need to be invoked to explain possible health risks to humans and wildlife. Initial reports of synergisms between estrogenic pesticides appeared to provide the much sought after link. However, it was the report from Arnold et al. [1996] claiming massive synergisms between binary combinations of pesticides that had the most profound and unexpected effect on the xenoestrogen field. This now infamous paper was later retracted as the results could not be reproduced [Ashby et al., 1997; Ramamoorthy et al., 1997], even by the original investigators [McLachlan, 1997]. The fallout from this unfortunate incident cast a long shadow on the relevance of mixture effects of xenoestrogens present at low levels.

Kortenkamp and Altenburger [1999] recently highlighted reasons why studies of mixture effects often lead to conflicting results. It is frequently assumed that the combined effects of a mixture can be computed simply by adding the effects of its individual components. However, this method produces unreliable results when dealing with agents that exhibit sigmoidal dose-response curves. A detailed explanation is given
in Chapter 4, along with a discussion of more applicable concepts that can be employed with agents that demonstrate non-linear concentration-response curves.

1.3 Thesis objectives

The work contained in this thesis can be broadly split into two parts, mirroring the bifunctional hypothesis that DNA damage and estrogen-like actions, often via the estrogen receptor, are involved in the increased incidence of endocrine related disorders. The bifunctional genetic-hormonal pathways described by Davis et al. [1997], formed a hypothetical mechanism by which exposure to estrogens and xenoestrogens might result in breast cancer (Figure 1.3). In effect, the genetic pathway involves metabolism of

![Diagram](image_url)

**Figure 1.3 - Hypothetical impact of steroid estrogens (and xenoestrogens) on genetic (blue arrows) and hormonal (black arrows) pathways of breast carcinogenesis.** Dashed arrows represent potential crossover between pathways.
estradiol to more reactive metabolites capable of redox cycling with the concomitant production of reactive oxygen species (ROS) that induce DNA damage. The hormonal pathway involves interaction with the estrogen receptor and potential interference with the classical receptor-activated pathways. It is assumed that the presence of xenoestrogens in this hypothesis will impact upon the availability and metabolism of the steroidal estrogens. The pathways combine to result in aberrant cell proliferation and potentially breast cancer.

It was the report by Nutter et al. [1991] describing the characterisation of DNA damage induced by 3,4 estrone-o-quinone that was the stimulus for the first part of this thesis. Using alkaline elution, Nutter and colleagues demonstrated the formation of DNA single strand breaks and alkali labile sites in MCF-7 breast cancer cells following treatment with a putative intermediate arising from metabolic conversion of E₂, 3,4-estrone-o-quinone. A combination of the fact that relatively high concentrations (>10 μM) of this o-quinone had been used and that more sensitive techniques for assessing DNA damage had recently been developed led us to question whether treatment with near physiological concentrations of the hormone, 17β-estradiol, might result in detectable levels of DNA damage (Chapters 2 and 3). The single cell gel electrophoresis (Comet) assay allows the detection of not only DNA SSBs and ALS, but also DNA double strand breaks and even oxidised bases. This assay, therefore, would give us a powerful tool with which we could investigate the DNA-damaging capability of 17β-estradiol and its 2- and 4-hydroxylated metabolites. As will be discussed in greater detail later, it has been proposed that the DNA-damaging species arising from the metabolism of estrogens are reactive oxygen species and thus the ability to detect oxidised bases may prove very desirable.

The second part of this thesis turns to the potential role of xenoestrogens in the increased incidence of detrimental health effects. I have previously referred to the prevalence of scientific assessments of xenoestrogen risks focussing on pure substances individually. By doing this, such studies fail to take into account the fact that environmental exposure is often to mixtures of such agents and this may be the reason why findings commonly indicate low risk. For the investigation of mixtures, it is important to be able to derive reliable predictions of the “toxicity” of multi-component mixtures from “toxicity” data on individual agents. Can we reliably use single agent concentration-response analyses to predict the effects of a complex mixture of those agents? Can the concepts that have been used successfully for predicting toxicity of
multi-component mixtures in other areas [Altenburger et al., 2000; Backhaus et al., 2000], be applied productively to the field of xenoestrogens? Chapters 4, 5 and, to some degree, 6 focus on these questions.

Typically, xenoestrogens are present in the environment at concentrations below which effects are observable, and in estrogenticity testing these agents demonstrate low potency in relation to 17β-estradiol. These factors are often paraded as the reasons why xenoestrogens are rarely conclusively linked with detrimental health effects [Safe et al., 1995b]. This argument is based upon the belief that in order to impact on, or modulate the effects of, the potent steroidal hormone, the xenoestrogen needs to be either as potent as the steroid or present at a very high concentration. The question arises as to whether combinations of weakly active xenoestrogens have the potential to enhance the actions of steroid hormones, even when each is present at low, ineffective concentrations. Chapter 6 begins to answer this complicated question at the simplest level, i.e. using a receptor based assay system.
Elevated levels of estrogens are known to produce adverse effects, such as embryotoxicity, teratogenicity and carcinogenicity [Roy et al., 1997]. The majority of these findings have only been observed in rodent species such as the Syrian hamster, however, there is growing concern that genetic alterations caused by estrogens may be involved in the induction of certain types of cancer in humans by this hormone.

Estrogen receptor (ER)-mediated events alone cannot explain the full spectrum of adverse effects of estrogens, which has led to the proposal that estrogens are genotoxic. A hypothesis [Davis et al., 1997], gaining much recent support suggests that the ER-mediated events and genotoxic events act together: Via their separate pathways they represent a conceivable mechanism leading to the development of a tumour; ER activation is thought to cause increased cellular proliferation by stimulating G1/S transition [Foster et al., 2001], while the induction of DNA damage might result in genetic alterations that cellular mechanisms may fail to recognise and repair prior to cell division.

The work presented in this and the following chapter concentrates on the ability of estrogens to be genotoxic. This chapter presents the proposed mechanisms before detailing the experimental approach and its validation.

### 2.1 Estrogen metabolism

17β-Estradiol and structurally similar steroid hormones are metabolically converted to hormonally less active water-soluble metabolites and then eliminated from the body in the urine and faeces. Metabolism includes oxidative metabolism, mainly hydroxylation, and conjugation, i.e. glucuronidation, sulfonation and O-methylation. Members of the cytochrome P450 family are the major enzymes catalysing β-nicotinamide adenine dinucleotide phosphate (reduced form) (NADPH*)-dependent oxidative metabolism of estrogens to multiple hydroxylated metabolites. Summarised in Figure 2.1 are the primary oxidative metabolic pathways for 17β-estradiol. These involve mainly
oxidations at the C-2, C-4 and C-16 positions of the steroid. It should be noted, however, that hydroxylated metabolites with oxidation at the C-1, C-6, C-7, C-11, C-14, C-15, C-17 and C-18 positions have also been detected in biological samples [Loke et al., 1959], isolated from in vitro incubations with enzyme preparations from animals [Maschler et al., 1983], or humans [Knuppen et al., 1965], although at much lower levels than the 2-, 4- and 16α-hydroxylated estrogen metabolites [Suchar et al., 1995].

The majority of the oxidative metabolism of 17β-estradiol occurs in the liver. However, some estrogen-metabolising isoforms of the cytochrome P450 family, that are usually expressed at low or undetectable levels in the liver, are selectively expressed in certain extrahepatic tissues. At least nine different isoforms of cytochrome P450 have been detected in the mammary gland of the female rat, some with estrogen-metabolising activity [Hellmold et al., 1995]. Hellmold and colleagues also showed that several isoforms are subject to endocrine and developmental regulation, a finding that was also observed in human breast tissue. They suggested that their observation may have implications for the activation of pro-carcinogens and for in situ metabolism of steroids and steroid antagonists.
2.1.1 2-Hydroxylation of estrogens

In the liver, 2-hydroxylation of estradiol or estrone to a catechol is a major metabolic pathway [Zhu et al., 1993; Suchar et al., 1995]. In rats, hepatic 2-hydroxylation of estradiol is catalysed by numerous isoforms of cytochrome P450, namely 1A2, 2B1/2B2, 2C6, 2C11, members of the 2D family and the 3A family [Ryan et al., 1984; Dannan et al., 1986; Watanabe et al., 1991; Suchar et al., 1996]. In humans, however, the main hepatic enzymes for 2-hydroxylation are cytochrome P450 1A2 and the 3A family [Kerlan et al., 1992; Shou et al., 1997]. These estrogen 2-hydroxylases lack specificity and therefore convert estradiol to 2-hydroxyestradiol (80-85%), and 4-hydroxyestradiol (15-20%) [Weisz et al., 1992].

NADPH-dependent 2-hydroxylation has been observed with microsomes prepared from extra-hepatic tissues such as the uterus [Paria et al., 1990], breast [Telang et al., 1991], placenta [Bui and Weisz, 1988], kidney [Zhu et al., 1994], brain and pituitary [Ball and Knuppen, 1978]. In MCF-7 human breast cancer cells treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the 2-hydroxylation of estradiol appears to be predominantly catalysed by cytochrome P450 1A1/1A2 [Spink et al., 1990], whereas, in rodent mammary glands cytochrome P450 3A4 is believed to contribute significantly to 2-hydroxylation [Hellmold et al., 1995].

The products of 2-hydroxylation of estradiol and estrone are capable of binding to ERα. However, both binding affinity and potency are very low compared to the parent steroids. 2-Hydroxyestrone (2-OH E1) has even been reported to partially antagonise the growth stimulatory effects of 17β-estradiol in MCF-7 breast cancer cells [Ball and Knuppen, 1980], and in rodents, treatment with indole-3-carbinol, an inducer of estradiol 2-hydroxylation, was seen to inhibit mammary preneoplasia and spontaneous mammary tumours [Bradlow et al., 1991].

Rapid conjugative metabolism of 2-OH E1 and 2-hydroxyestradiol (2-OH E2), i.e. O-methylation, glucuronidation and sulfonation, followed by urinary excretion ensure that under normal conditions systemic levels of the unconjugated metabolites are kept very low [Emons et al., 1987]. In fact, when compared with the estrogen products of 4-hydroxylation, the 2-hydroxyestrogens have a faster rate of metabolism by catechol-O-methyltransferase-catalysed O-methylation, a more rapid clearance in vivo, and possess weaker hormonal potency in estrogen target tissues.
2.1.2 4-Hydroxylation of estrogens

In the liver, 4-hydroxylation of estradiol or estrone represents only a minor metabolic pathway (approximately 15% of 2-hydroxylation). Cytochrome P450 1A2, 2B1/2, and the 3A family catalyse 4-hydroxylation of estrogens in the rodent liver [Dannan et al., 1986; Suchar et al., 1996], whilst in human liver microsomes the cytochrome P450 3A family plays a major role [Kerlan et al., 1992].

Although only a minor pathway for catechol formation in the liver, studies have shown that 4-hydroxylation of estradiol is a dominant pathway in some extrahepatic target tissues. Rat pituitary has little or no 2-OH E$_2$ formation, yet it expresses an estradiol 4-hydroxylase activity [Bui and Weisz, 1988]. Similar findings have been reported for human uterine myometrial and myoma tissues [Liehr et al., 1995]. Cytochrome P450 1B1 is an important enzyme for the 4-hydroxylation of estradiol in the human breast and uterus [Liehr et al., 1995], expressing the human P450 1B1 gene in Saccharomyces cerevisiae produced an enzyme that catalysed both 2- and 4-hydroxylation of estradiol, but with a more than five-fold higher turnover in favour of 4-hydroxylation [Liehr et al., 1995; Hayes et al., 1996].

In direct contrast with 2-OH E$_2$, 4-hydroxyestradiol (4-OH E$_2$) is similar to 17β-estradiol in its ability to bind and activate ERα, and in fact the dissociation rate for 4-OH E$_2$ is lower than that for the parent hormone, suggesting a longer association with the ER [Martucci and Fishman, 1976].

The specific formation of 4-hydroxylated estrogens in organs expressing cytochrome P450 1B1 is important, because 4-OH E$_2$ is as carcinogenic as E$_2$ in the hamster kidney tumour model [Liehr et al., 1986], and nine times more carcinogenic in the mouse model [Newbold and Liehr, 2000].

2.1.3 16α-Hydroxylation of estrogens

A strong catalytic activity for estrone 16α-hydroxylation in humans was shown for cytochrome P450 3A4/5, isoforms detectable in breast tissue [Huang et al., 1998]. Apart from this, little is known about the enzymes involved in man. The male specific cytochrome P450 2C11 isoform from male rat liver microsomes has 16α-hydroxylation
activity [Suchar et al., 1995], whilst numerous prototype liver microsomal enzyme inducers have been shown to result in little or no induction of 16α-hydroxylation [Suchar et al., 1996].

Like 4-hydroxylated estrogens, 16α-hydroxylated estrogen metabolites retain potent hormonal activity via ERα [Fishman and Martucci, 1980]. Some groups have reported an ability of 16α-hydroxyestrone (16α-OH E1) to stimulate MCF-7 breast cancer cell growth to levels comparable to the parent hormone [Lottering et al., 1992]; or a significant increase in unscheduled DNA synthesis in mouse mammary epithelial cells [Telang et al., 1992]. 16α-OH E1 causes prolonged growth by virtue of its ability to bind covalently to the ER [Swaneck and Fishman, 1988]. This formation of stable adducts may not be confined to the ER, since a reversible reaction between 16α-OH E1 and an amino group will form a Schiff base which will further form a stable 16-keto-17β amino estrogen via a Heyns arrangement [Miyairi et al., 1991]. Although the potential to bind covalently with DNA can be inferred from features of the chemical structure of 16α-OH E1, it remains to be shown experimentally.

It has been suggested that increased formation of 16α-hydroxyestrogen metabolites, relative to 2-hydroxylated estrogen metabolite formation, is associated with an increased risk for mammary cancer in mice and humans [reviewed in Zhu and Conney, 1998], but as the authors of the review note:

These studies have not yet received sufficient confirmation by other investigators using different experimental settings.

2.2 Estradiol as a genotoxic carcinogen - proposed mechanisms

It is generally believed that metabolic activation of estradiol leading to the formation of catechol estrogens is a prerequisite for its hypothesised genotoxic activity. Some groups have proposed that the steroid estrogens may generate reactive intermediates, particularly arene oxides and quinones/semiquinones during their metabolism [Metzler et al., 1980; Liehr and Roy, 1990]. The previous sections dealt with the metabolism of estradiol to catechol estrogens, and it is at this point that the description turns to their metabolic activation to reactive intermediates.
2.2.1 Metabolic activation of catechol estrogens

Catechol estrogens (CEs) can undergo metabolic one-electron redox cycling between the hydroquinone (catechol) and quinone forms via the chemically reactive estrogen semiquinone. The process (illustrated for 4-hydroxyestradiol in Figure 2.2) consists of the oxidation of the CE by organic hydroperoxide-dependent cytochrome P450 1A enzymes or other peroxidases, and the reduction of the estradiol quinone to the CE by NADPH-dependent cytochrome P450 reductase or NADH-dependent cytochrome b5 reductase [Liehr et al., 1986]. Intermediate in both the oxidation and reduction reactions is the estrogen semiquinone free radical.

![Figure 2.2 - Proposed pathway of redox cycling of estrogens (shown for 4-hydroxylated estradiol). The catechol estrogen may undergo metabolic redox cycling to the semiquinone and quinone and each of these steps may produce superoxide radicals that are reduced to hydrogen peroxide by superoxide dismutase. The potential of estrogen metabolites to damage cells is potentiated by redox cycling and by the initiation of free radical reactions (see text for details). Adapted from Nutter et al., 1994.](image)

Reactive oxygen species may be generated from two pathways subsequent to the formation of the catechol estrogens; (i) the semiquinone intermediate may react with molecular oxygen to form quinone and superoxide radicals [Roy and Liehr, 1988], and (ii)
the non-enzymatic redox couples between copper ions and CEs generates reactive oxygen radicals [Mobley et al., 1999]. Thus, two mechanisms exist by which continuous production of free radicals and other reactive oxygen species may occur from the cycling of possibly small amounts of CE substrate. The duration of such redox cycling will be dependent on the availability of the catechol substrate and cofactor or metal ion for the oxidation step of the cycle.

2.2.2 DNA damage profile induced by estrogens

A number of reports exist describing DNA damage resulting from exposure to estrogens or their metabolites in both *in vitro* and *in vivo* test systems. The forms of DNA damage described include strand breaks, 8-hydroxylation of guanine bases and both direct and indirect estrogen DNA adducts.

DNA single strand breaks assumed to be free-radical mediated have been seen in MCF-7 human breast cancer cells in culture induced by 3,4-estrone-o-quinone [Nutter et al., 1991; 1994], and in plasmid DNA exposed to 2-hydroxyestradiol and Cu(II)sulfate [Li et al., 1994]. *In vivo* exposure of Syrian hamsters to E₂ or 4-OH E₂ resulted in the formation of DNA single strand breaks in the kidneys [Han and Liehr, 1994]. The significance of the Han and Liehr findings became even greater when it was shown that months after treatment the kidneys developed neoplasms.

Elevated concentrations of 7,8-dihydro-8-oxo-guanine have also been detected as a result of the reaction between hydroxyl radicals and guanine bases. These observations have been described following incubation of DNA with catechol estrogens and Cu(II)sulfate [Mobley et al., 1999], with 4-hydroxylated estrogen metabolites and a microsomal activating system [Han and Liehr, 1994b], and treatment of Syrian hamsters with diethylstilbestrol (DES) [Roy et al., 1991]. Malins et al. [1993; 1995], found corresponding increases in hydroxyl radical damage to DNA in human mammary tissue of breast cancer patients relative to controls. Further confirmation of the damage being resultant from estrogen-induced free radical action, damage to cellular macromolecules other than DNA such as protein oxidation [Winter and Liehr, 1991], and lipid peroxidation [Wang and Liehr, 1995], has also been described.
Decomposition of the products from the aforementioned lipid peroxidation can form reactive aldehydes capable of DNA adduct formation [Wang and Liehr, 1995]. Again, low levels of corresponding adducts have been identified in mammary DNA of breast cancer patients [Wang et al., 1996].

In addition to this somewhat indirect DNA adduct formation, estrogen metabolites themselves have also been shown to form DNA adducts. The intermediate products of the one-electron oxidations of the catechol estrogens are capable of covalent binding to DNA in vitro. They are Michael acceptors, and as such possess reactivity to various nucleophilic sites within the DNA bases, particularly the amino groups [Akanni and Abul-Hajj, 1997]. The potential consequence of the formation of estrogen-DNA adducts differs significantly depending upon the original catechol estrogen. Specifically, the purine adducts of estrone-3,4-quinone, so-called depurinating adducts, are unstable and readily decompose to apurinic sites, which are believed to be carcinogenic. Cavalieri et al. [1997], found that estrogen-3,4-quinones bound exclusively to the N7 of guanine, resulting in destabilisation of the N-glycosidic bond and subsequent depurination. In contrast, estrone-2,3-quinone binds mainly to the exocyclic amino groups to form DNA adducts that are highly stable and retain the deoxyribose moiety, and this is seen as consistent with 2-hydroxylated estrogens being only weakly carcinogenic. The description of this adduct pattern led to the proposal of a mechanism of carcinogenesis by unstable adduct formation of 4-hydroxylated estrogens, induction of gene mutations and subsequent tumour initiation [Cavalieri et al., 1997].

2.2.3 Chromosomal and genetic mutations associated with estrogens

Using classical gene mutation assays, such as the Ames bacterial reversion test, neither estradiol nor its catechol metabolites induced point mutations [Lang and Redmann, 1979], and based on this failure estrogens were classified as nonmutagenic and nongenotoxic [Nandi, 1978; Tsutsui et al., 1983]. This is in clear disagreement with the DNA damage shown to be induced following treatment with estrogens (as discussed above) and known to be potentially mutagenic.

Recent findings of inactivation of the gpt transgene of the Chinese hamster G12 cells by E₂ or DES [Su and Klein, 1999], of 4-hydroxyestrone and 16α-hydroxyestrone induced methotrexate resistance in MCF-7 breast cancer cells (also seen to a lesser
degree with 2-hydroxyestrone and estradiol) [Thibodeau et al., 1998], and mutation of the hprt gene in Chinese hamster V79 cells [Rajah and Pento, 1995] point to estrogen-induced gene mutations.

Estradiol has also been shown to induce aneuploidy (numerical chromosomal aneuploidy) [Tsutsui et al., 1990] and structural chromosomal changes (deletions, inversions and translocations) [Jones and Hajek, 1995], which alone may be insufficient for tumours to develop, but may be indicative of, and part of, a larger pattern of covalent damage to the cell’s DNA.

The genetic lesions described, i.e. DNA microsatellite instability, DNA sequence deletions, gene amplification, chromosome aberrations and aneuploidy have been proposed as the basis of most human cancers [Lengauer et al., 1998].

Although the data outlined above clearly demonstrate that the natural hormone estradiol is a carcinogen in both humans and animals, many gaps exists in our understanding of the mechanistic events resulting in hormone-associated cancer. For instance, the demonstration by Nutter et al. [1991], that estrogen metabolites could induce DNA damage in MCF-7 breast cancer cells involved exposure to extremely high levels (upwards of 10 µM), of the metabolite. We question whether this mechanism is relevant when beginning with “near-physiological” concentrations of the parent compound, 17β-estradiol. Can DNA strand breaks be seen when MCF-7 cells are exposed to 17β-estradiol at concentrations approaching physiological levels, i.e. below 1 µM?

2.3 DNA damage detection methods

A number of techniques are available which enable the detection of DNA damage. The major limitation with all such assays is sensitivity. To be useful it must allow measurement of at least one modification in $10^4$ - $10^6$ normal bases within a few micrograms of DNA. Two approaches can be taken: In the first, the DNA is kept intact and the lesions are measured either by immunological methods or by nicking activity of DNA repair enzymes in association with sedimentation and gel-sequencing techniques that quantify the number of breaks; the second requires either quantitative acidic
hydrolysis or enzymatic digestion. Separation of the compounds of interest can be achieved by using HPLC and GC, and detection by methods such as mass spectrometry.

2.3.1 32P-postlabeling assays

These methods were developed to measure carcinogen-DNA adducts [Randerath et al., 1981]. Damaged DNA is first digested enzymatically to nucleoside 3'-monophosphates or very short oligonucleotides. Using 32P-labeled ATP and phage T4 polynucleotide kinase the digested products are enzymatically radiolabelled before analysis of the products with two dimensional thin layer chromatography or HPLC. The fact that 32P-labeled ATP is commercially available at high specific activity makes this approach highly sensitive, the downside being that radioactive compounds are involved.

2.3.2 Alkaline elution

Alkaline elution techniques measure the rate of DNA elution through a filter membrane under alkaline conditions. Very little of the undamaged DNA is normally able to pass the filters. The amount of DNA single-strand breaks (SSB) or lesions converted to SSB under alkaline conditions is estimated on the basis of increases in the amounts of DNA fragments found in eluates. This approach requires a relatively large number of cells and ignores the critical importance of intercellular differences as samples have to be pooled. Slight variations in buffer composition, pH and temperature have been shown to have considerable influence on the elution rate of DNA and therefore affect this assay's sensitivity [Leroy et al., 1996]. Leroy et al., also called into question its suitability for certain forms of DNA damage and certain cell types.

2.3.3 Single cell gel electrophoresis (Comet) assay

The single cell gel electrophoresis assay, also known as the Comet assay, is a rapid, simple, visual, and sensitive technique for detecting DNA damage at the level of the single cell. It was first described by Östling and Johanson [1984], and involved
embedding cells in agarose on a microscope slide before lysing the cells with detergents and high salt. The naked DNA (the nucleoid), was then electrophoresed under neutral conditions and cells with an increased frequency of DNA double-strand breaks (DSB) would display an increased migration of DNA towards the anode, visualised by measuring the intensity of ethidium bromide staining.

By utilising neutral conditions the assay was clearly limited, a fact that was understood by Singh et al. [1988], who introduced electrophoresis of the microgels under alkaline (pH > 13) conditions. This alkaline version of the Comet assay represents a major improvement, allowing the detection of frank SSB, SSB associated with incomplete excision repair sites, and alkali-labile sites (ALS). The increased sensitivity offered by this improved protocol is due to the fact that the majority of genotoxic agents induce orders of magnitude more SSB and ALS than DSB.

The Comet assay offers numerous advantages over the assays described above: detection of low levels of DNA damage; small number of cells required; low costs; ease and speed of application; and the ability to conduct studies with a relatively small amount of test agent. The Comet assay represented the assay of choice to meet our requirements, as we expected to see only very low levels of DNA damage, if any, following exposure to 17β-estradiol. This chapter will concentrate on the assay's validation, both in terms of experimental protocol and data analysis.

2.4 Experimental procedure

The findings of Nutter et al. [1991], represent the most substantial report of estrogen metabolites causing DNA damage and so we considered it important to investigate the parent hormone in the same cell line i.e. MCF-7 breast cancer cells. Since their initial derivation from a pleural effusion of a breast carcinoma [Soule et al., 1973], the MCF-7 cell line has been widely used in mechanistic studies of hormone-associated cancer and as a result are well characterised.

In terms of suppliers, unless stated otherwise, chemicals were purchased from Sigma Chemical Co, Dorset, U.K.
2.4.1 Routine cell culture

MCF-7 breast cancer cells (obtained from M. Dufresne, University of Windsor, Ontario, Canada) were maintained in 25 cm² cell culture flasks (Greiner Labortechnik, Frickenhausen, Germany) in α-MEM with GLUTAMAX-I (Invitrogen, Paisley, UK) supplemented with 5% heat inactivated fetal calf serum (FCS, Invitrogen) in a humidified incubator at 37°C, 5% CO₂. Cells were passaged at approximately 70% confluence over a maximum of twenty passages, before resurrecting cells from frozen stocks. Cells were routinely tested for mycoplasma.

2.4.2 Preparation and treatment of cells for the Comet assay

For validation purposes, the Comet assay was performed on cells exposed to UV-C radiation and for this reason it was necessary to grow the cells in petri dishes with lids that could be removed, enabling the UV-C radiation to reach the cells.

One 70% confluent 25 cm² flask of MCF-7 cells was washed with 5 mL Hanks’ Balanced Salt Solution without phenol red (HBSS, Invitrogen), before the addition of 660 μL 0.25% trypsin-EDTA. Following incubation at 37°C for 3 minutes the cells were detached by pipetting 20 mL α-MEM over the seeded surface of the vessel. Petri dishes (3.5 cm diameter, Greiner) were seeded with 1 x 10⁶ cells in 3 mL α-MEM. Following seeding, exposure to light was kept to a minimum, as this has been shown to induce DNA damage measurable in the Comet assay.

Cells were treated 72 hours after seeding. UV irradiation of MCF-7 cells, after removal of growth medium, was carried out using a germicidal lamp emitting predominantly at 254 nm, at a dose rate of 7.6 J/m²/s (UVP, California, USA). Following exposure, 3 mL fresh α-MEM was added to the cells. They were then placed in an incubator (37°C) for 1 hour.
2.4.3 Obtaining a single cell suspension and checking cell viability

As stated previously, the key advantage of the Comet assay is its ability to collect data at the level of the individual cell. To enable analysis of the results, it is imperative that a single cell suspension be obtained before beginning the Comet assay protocol.

Following UV-C irradiation and the period of incubation for DNA damage processing, the medium was removed and the cells washed with 3 mL HBSS prior to the addition of 400 μL 0.25% trypsin-EDTA for 3 minutes. Cells were desegregated by the addition of 3 mL α-MEM and repeated gentle pipetting. Cells were pelleted (200 × g, 5 minutes) and the medium replaced with 1.25 mL phosphate buffered saline (PBS, calcium and magnesium free, pH 7.4).

At this point cell viability was assessed using trypan blue exclusion, a rapid test for gross damage. Briefly, a 250 μL aliquot of cell suspension was mixed with an equal volume of 0.5% trypan blue solution. Within 5 minutes the suspension was introduced into an improved Neubauer haemocytometer before enumerating the number of viable and non-viable cells. Under this assessment a non-viable cell is assumed to have a permeable membrane and therefore, will appear with a darkly stained nucleus. From this procedure the viability of the cells and the cell number have been determined as well as confirming that a single cell suspension has been achieved. The optimum concentration of cells was between 5 \times 10^5 and 1 \times 10^6 cells/mL and suspensions were diluted accordingly. After these steps, the cells were used immediately for the Comet assay.

2.4.4 The Comet assay

Once a suspension of cells is obtained, the basic steps of the assay involve; embedding of cells in agarose upon a microscope slide; lysis of cells to remove membranes and proteins to leave behind DNA; unwinding of DNA in alkaline conditions which also allows alkali labile sites to be expressed as single strand breaks; electrophoresis under alkaline conditions; neutralisation; and finally DNA staining and comet analysis or scoring. The adopted protocol, described below, was modified from Singh et al. [1988], and was carried out under UV-free lighting conditions.
Fully frosted glass microscope slides (TAAB Laboratories, Aldermaston, U.K.), were cleaned in ethanol and allowed to dry, before coating the frosted side with 100 μL normal melting point agarose (1% w/v, high strength analytical grade agarose, BioRad Laboratories, CA, USA) prepared in phosphate buffered saline (PBS, pH 7.4). Slides were allowed to dry before use. By precoating the slides in this way, the subsequent layer of agarose containing cells will be more stable and therefore less likely to detach from the slide during the lysis and electrophoresis stages.

From the previously prepared cell suspension, a 10 μL aliquot was mixed with 85 μL of 0.75% (w/v) low melting point agarose (NuSieve GTG Agarose, FMC Bioproducts, ME, USA), at 37°C, similarly prepared in PBS and the whole immediately pipetted on to a precoated slide. A 24 x 24 mm No. 1 coverslip was placed over the gel to spread it evenly over the slide before placing the slide on an ice-tray for 30 s and then removing the coverslip. Duplicate slides were prepared.

After the agarose gel had solidified the slides were immersed horizontally in freshly prepared cold lysis solution (Incomplete lysis solution; 2.5 M NaCl, 100 mM EDTA, 10 mM Trizma base, adjusted to pH 10.0, can be prepared and stored at 4°C for up to 6 weeks, and to which is added 1% (v/v) Triton X-100 and 10% (v/v) DMSO just prior to use).

Following lysis for 3 hours at 4°C, the slides were placed in a double row in a 260 mm wide horizontal electrophoresis tank filled with fresh electrophoresis buffer (1 mM EDTA, 300 mM NaOH, pH > 13.0 at 4°C). The field current is affected at the edges of the slides, altering the electrophoresis conditions in these areas of the tank. To minimise these effects, blank slides were used for the outer edges. Prior to electrophoresis, the slides were incubated in the electrophoresis buffer for 20 minutes to produce single stranded DNA and to express ALS as SSB.

After alkaline unwinding, the single stranded DNA in the agarose gels was electrophoresed, using the same buffer, for 20 minutes at 25 V (0.7 V/cm), adjusting the level of the buffer to maintain a 300 mA current. To ensure greater reproducibility, the buffer temperature over the course of the electrophoresis was kept below 15°C.

Prior to staining and evaluation, the alkali in the gels was neutralised by rinsing three times for 5 minutes each in 0.4 M Tris-HCl, pH 7.5, 4°C. If slides were not analysed immediately they were dehydrated by immersion in increasingly concentrated ethanol solutions i.e. starting with 70% ethanol for 5 minutes, then 80% ethanol, and
then absolute ethanol. Slides could then be stained and analysed at a later date, without the slides degrading in any way.

### 2.4.5 Quantification of the Comet assay

Ethidium bromide stained slides were evaluated on a Leitz Axiophot 20 with epifluorescent capabilities projecting images onto a sensitive monochrome CCD camera. Image analysis was carried out using Kinetic Komet 3.0 software (Kinetic Imaging Ltd., Liverpool, U.K.). For each cell analysed, this software computes an integrated image profile and then, according to pre-set criteria, defines the cell components, i.e. head and tail (see Figure 2.3). Based on this, a number of metrics can be measured such as length of DNA migration, percentage of migrated DNA, tail moment, etc. For robust analysis, at least 50 cells were scored in each of two replicate slides.

![Figure 2.3 - Example of a comet (top), and digitised fluorescent intensity profile (bottom). Software algorithms break down the intensity profile into head and tail components, before calculating a number of comet parameters.](image)

Although still an area of greatly varying opinions, the data presented herein utilises the metric of tail moment, or Olive tail moment, as first proposed by Olive et al. [1990], which we believe represents a better reflection of the level of damage than other
measures. Tail moment is the product of the tail length (measured from the trailing edge of the head) and the fraction of total DNA in the tail.

It has been suggested that tail length may not fully reflect the extent of damage in samples exposed to powerful DNA damaging agents for prolonged time periods [Olive and Durand, 1992]. During electrophoresis, there is a defined limit to the distance a fragment of DNA can migrate in the time, representing a maximal tail length. Thus, above a certain level of damage any increase in DNA damage will not be accompanied by the expected increase in comet tail length. Increased damage is also visualised as an increased level of fluorescence in the tail of the comet compared to that of the head, i.e. the fraction of migrated DNA. By combining these two measures in the Olive tail moment it is possible to minimise the effect of electrophoresis duration on the assay outcome.

2.4.6 Data analysis and statistical approaches

Inherent to the Comet assay is the assessment of heterogeneous populations of cells without the loss of individual cell data. For each treatment a population distribution for the tail moments can then be constructed, thus allowing an immediate assessment of the study findings by comparing the control distribution with those of the treatment groups.

To test statistically whether two treatment groups have been drawn from the same population, the Wilcoxon-Mann-Whitney test was performed. This is one of the most powerful of the non-parametric tests, and was chosen as an alternative to the $t$ test. The assumptions associated with the $t$ test make it inappropriate for testing Comet assay data, in particular the condition that observations be drawn from a normally distributed population. The Wilcoxon-Mann-Whitney test was preferred above the one-tailed Kolmogorov-Smirnov two-samples test as the power-efficiency of the former is higher with larger sample sizes [Siegel and Castellan, 1988].

For an experiment with two treatment groups, i.e. a control ($c$) and a dosed ($d$) group, the data (100 tail moments per dose) were ranked together for both groups with the lowest being assigned 1. The ranks ranged from 1 to 200, with tied observations being assigned the average of the tied ranks. For each treatment group the ranks were summed ($W_c$ and $W_d$). As the sample size is large, the probability associated with a
value as extreme as the observed value of $W_d$ was determined by computing the normal approximation using Eq. (2.1);

$$z = \frac{W_d + 0.5 - m(N + 1)/2}{\sqrt{mn(N + 1)/12}}$$

where $m$ and $n$ are the number of tail moments in the control and treated groups, respectively, and $N$ is the total number of ranks. The null hypothesis that treatment had no effect upon tail moments was rejected if the observed value of $W_d$ had an associated probability equal to or less than $\alpha$ (in our case $P < 0.05$).

Replicate experiments carried out on different occasions were analysed using two tests, firstly the Kruskal-Wallis one-way analysis of variance by ranks (Eq. (2.2));

$$KW = \left[ \frac{12}{N(N + 1)} \sum_{j=1}^{k} n_j \bar{R}_j^2 \right] - 3(N + 1)$$

where $k =$ number of replicate datasets; $n_j =$ number of tail moments in the $j$th dataset; $N =$ number of tail moments in the combined sample (the sum of the $n_j$'s); $R_j =$ sum of the ranks in the $j$th dataset; $\bar{R}_j =$ average of the ranks in the $j$th dataset; $\bar{R} = (N + 1)/2$ or the average of the ranks in the combined sample; and the summation is across the $k$ datasets. In this way it was possible to test whether controls of different experiments differed significantly, and likewise for identical treatments in replicate experiments. If the null hypothesis, i.e. there being no difference between controls, is accepted, results were assumed to have been drawn from the same population and data were pooled for analysis using the Wilcoxon-Mann-Whitney test.

It has previously been reported that distributions of tail moments obey a chi-square ($\chi^2$) distribution [Bauer et al., 1998]. As will be seen in the section below, the histograms are often asymmetrical, especially in samples displaying low levels of DNA damage, clearly not favouring the use of the Gauss distribution. We studied the application of fitting the $\chi^2$ distribution to our histograms.
The \( \chi^2 \) density function is defined by Eq. (2.3);

\[
P(\chi^2) = \frac{1}{2^\frac{df}{2} \Gamma\left(\frac{df}{2}\right)} \left(\chi^2\right)^{\frac{df}{2} - 1} e^{-\frac{1}{2} \chi^2}
\]  

(2.3)

\( df \) is the degree of freedom, which is the only parameter of this distribution. The degree of freedom of a given \( \chi^2 \)-fit to the data not only approximates the median value of the distribution, but also defines the shape of the distribution, encompassing a measure of dispersion and skewness.

In order to quantify the asymmetry of the distribution, the “skewness” is calculated from the second and third moments of the sets of raw data of the tail moment: Using the \( k \)th moments of a distribution;

\[
m = \frac{1}{z} \sum_{i=1}^{z} (x_i - \bar{x})^k
\]

the skewness can be calculated as;

\[
\text{skewness} = \frac{m_3}{(m_2)^{\frac{3}{2}}}
\]

where \( z \) = number of data, \( x_i \) = tail moments, \( \bar{x} \) = mean value of the tail moment.

As the \( \chi^2 \)-distribution tends to be skewed for low values of \( df \), the limits of error are also asymmetrical and can be defined by

\[
\text{Prob}\left(\chi^2_{df;\alpha/2} \leq \chi^2 \leq \chi^2_{df;1-\alpha/2}\right) = \alpha
\]

(2.4)

Tail moment data were transformed into histograms normalised to unit area using SigmaPlot for Windows version 5.0 (SPSS Inc., CA, USA), before fitting the \( \chi^2 \) distribution. This was achieved using the mathematical non-linear regression curve-fitting capabilities of the program (The appropriate formulas were kindly provided by Dr Eckhard Bauer, Germany). In subsequent tables and graphs the parameter used is the result of the \( \chi^2 \)-fit to the data, i.e. degree of freedom (\( df \)).
2.5 Results

UV-C is a consistent DNA damaging agent which we used to optimise protocols and also allowed inter-experimental reproducibility to be investigated. To facilitate this, Comet assay experiments were run three times, on separate occasions.

2.5.1 Effect of UV-C exposure on MCF-7 human breast cancer cells

Exposure of MCF-7 breast cancer cells, in logarithmic growth, to UV-C radiation induced DNA damage at levels detectable using the alkaline version of the Comet assay (Figures 2.4, 2.5 and 2.6). Cells irradiated with 0, 114, 228 and 342 J/m² UV-C (equivalent to 0, 15, 30 and 45 seconds exposure) and processed following 1 hour post-treatment incubation, for the cells to begin processing the DNA damage, exhibited a dose-dependent increase in DNA damage.

Control samples, i.e. cells not exposed to UV-C radiation, showed relatively low levels of DNA damage ($df = 2.57$, given by the $\chi^2$-fit to data), although there were some damaged cells. The proportion of these was extremely low. Levels of DNA damage appeared to plateau between 30 and 45 seconds treatment ($df = 33.26$ and 35.32, respectively).

![Figure 2.4 - Experiment 1 tail moment histograms following MCF-7 exposure to UV-C for increasing periods of time. Histograms are superimposed with the fitted curves (blue lines) calculated by the function of the $\chi^2$ distribution. (Dose rate: 7.6 J/m²/s).](image)
Reproducibility was very encouraging, as demonstrated by the comparability between the degrees of freedom gained by the $\chi^2$-approximation to the distributions of tail moments ($df$) for similar exposures from the three replicate experiments (Table 2.1).

There were no significant differences ($P > 0.05$) in tail moments between the three populations of control cells, given by the Kruskal-Wallis one-way analysis of variance by ranks, these cells were pooled as were the comet data from samples given
identical treatments. At only 15 seconds exposure the tail moments were statistically increased compared to controls (Wilcoxon-Mann-Whitney test, P < 0.05), whereas there was no difference between the 30 and 45 second exposure samples (P > 0.05).

Table 2.1 - The degrees of freedom gained by a $\chi^2$-approximation of the distribution of tail moments of replicate Comet assays of MCF-7 cells exposed to UV-C radiation.

<table>
<thead>
<tr>
<th>Dose (s)</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.51</td>
<td>2.49</td>
<td>2.71</td>
</tr>
<tr>
<td>15</td>
<td>22.31 $^d$</td>
<td>22.76 $^d$</td>
<td>23.05 $^d$</td>
</tr>
<tr>
<td>30</td>
<td>33.00 $^d$</td>
<td>31.94 $^d$</td>
<td>34.83 $^d$</td>
</tr>
<tr>
<td>45</td>
<td>35.30 $^d$</td>
<td>34.72 $^d$</td>
<td>35.93 $^d$</td>
</tr>
</tbody>
</table>

$^a$ degrees of freedom as calculated by fitting the $\chi^2$ distribution to tail moment histograms

$^b$ period of exposure to UV-C radiation followed by 1 hour post-treatment incubation (dose rate 7.6 J/m$^2$/s)

$^c$ replicate experiments carried out on different occasions

$^d$ significant DNA damage compared to controls given by Wilcoxon-Mann-Whitney test, P < 0.05

When the $\chi^2$-fit parameter ($df$) is plotted against dose along with the limit of error (calculated using Eq. (2.4)), it is possible to see the level of asymmetry in the data (Figure 2.7).

Figure 2.7 - Dose-dependent increase in DNA damage, described by the degree of freedom ($df$) and 95% limit of error, calculated from $\chi^2$ distribution curve fitting to tail moments of UV-C treated cells. Squares are the true value of tail moment and lines are the limits of error. Dose rate: 7.6 J/m$^2$/s.
2.5.2 UV-C irradiation of confluent MCF-7 cells

To study whether a less metabolically active and less rapidly dividing population of cells would exhibit a different DNA damage profile following UV-C exposure, approximately 100% confluent MCF-7 breast cancer cells were exposed and processed (Figure 2.8).

Processing of the damage induced by UV-C exposure was clearly slower in confluent cells than in cells actively dividing. At all three exposure doses the levels of damage observed were similar, $df$ being between 9.38 and 9.58. These, however, were much lower than even the lowest exposure in 70% confluent cells ($df = 22.71$), but even this increase in tail moment was statistically significant (Wilcoxon-Mann-Whitney test, $P < 0.05$).

![Figure 2.8 - Effect of increasing exposure to UV-C radiation on DNA damage in confluent MCF-7 cells. Histograms represent pooled data from two experiments, $df$ is the degrees of freedom from the $\chi^2$-distribution fit to the data (blue line).](image)

2.5.3 Are apoptotic cells distinguishable in the Comet assay?

It is clearly necessary to show that the damaged cells observed in the Comet assay are not apoptotic. This would work against our hypothesis that estradiol induces DNA damage which, instead of being considered irreparable and sending the cell into the
apoptotic cascade, is incorrectly repaired leading to a mutation becoming fixed in the genome.

It has been frequently reported that apoptotic cells are recognisable in the Comet assay as they take on a characteristic shape following electrophoresis, often referred to as a “hedgehog” [Fairbairn et al., 1994]. The extent of DNA fragmentation in apoptotic cells is so great that the DNA migrates several times the length of the original undamaged comet head, in fact the majority of the cell’s DNA will undergo migration, leaving behind a very small comet head. However, the levels of fragmentation due to apoptosis are dependent on cell line and damaging agent [Fairbairn et al., 1995]. Hence, for the cell line in use here we needed to confirm that apoptotic cells would be easily detected.

MCF-7 cells were seeded at a density of $1.5 \times 10^5$ cells per T25 flask and allowed to attach for 48 h, at which point apoptosis was induced by either serum withdrawal or heat-shock treatment. In the first case, medium was changed to phenol red-free DMEM supplemented with L-glutamine (200 mM) and left for a further 36 h. In the latter, flasks were placed at 50°C for 5h. Following treatment cells were processed for either the Comet assay or the Annexin V / propidium iodide analysis.

For the Comet assay, cells from the supernatant were centrifuged and then resuspended in PBS, without the need for the trypsinisation stage, at which point they were processed in the Comet assay as normal (see section 2.4.4).

Annexin-V stains phosphatidylserine (PS), which is normally found on the inner side of the plasma membrane. In the early stages of necrosis and apoptosis it is translocated to the outer layer and thus exposed to Annexin-V. By combining Annexin-V with a stain such as ethidium bromide or propidium iodide (PI), differentiation between apoptotic and necrotic cells becomes possible due to the fact that apoptotic cells will exclude the DNA stain. Cells that have translocated PS to the outer membrane appear green, the necrotic cells are differentiated from the apoptotic by the fact that they also fluoresce red (filter >600 nm) due to the intercalation of PI with the DNA.

Staining was carried out according to manufacturers instructions (Annexin-V-FLUOS Staining Kit, Boehringer Mannheim). Firstly, $1 \times 10^6$ cells were washed in PBS and centrifuged at $200 \times g$ for 5 min, before resuspending the pellet in 100 μL of staining solution (Annexin-V-fluorescien, propidium iodide and HEPES) on ice for 10-
15 min. Cells were visualised using fluorescence microscopy (488 nm excitation and a 515 nm long-pass filter for detection).

Serum withdrawal gave a high proportion of apoptotic cells after 36 h, as seen by Annexin-V-fluorescin binding to cells with no PI intercalation with DNA, the level of non-viable cells was low, being approximately 15%, which corresponded agreeably with the number of necrotic cells observed (Table 2.2).

Table 2.2 - Proportion of apoptotic and necrotic cells observed using Annexin-V-fluorescin and propidium iodide, following either serum withdrawal or heat shock.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% viable (Trypan blue)</th>
<th>% necrotic (Annexin-V/PI +)</th>
<th>% apoptotic (Annexin-V +)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum withdrawal</td>
<td>84</td>
<td>&lt; 10</td>
<td>&gt; 85</td>
</tr>
<tr>
<td>Heat-shock</td>
<td>15</td>
<td>&gt; 95</td>
<td>&lt; 5</td>
</tr>
</tbody>
</table>

* see text for details of protocols.

Heat-shocking the cells yielded almost exclusively necrotic cells, which was evident from trypan blue exclusion giving 15% viability (Table 2.2). This was further substantiated by the Annexin-V/PI analysis of the cells, which demonstrated that the cells had translocated PS to their exterior and that membrane integrity was lost, i.e. both stains were visualised (Figure 2.9).

Figure 2.9 - Appearance of apoptotic and necrotic cells using Annexin-V/Propidium iodide stains. Apoptotic cell fluorescing green (left), due to Annexin-V excitation (515 nm) following binding to externalised PS residues, no PI staining observed (>600 nm). Two necrotic cells exhibiting both Annexin-V binding and PI intercalation, (centre and right photomicrographs, respectively). Magnification x400.

Having established that the two regimens, namely serum withdrawal and heat-shock, induced almost exclusively apoptosis and necrosis, respectively, the appearance of these cells was investigated in the Comet assay. The apoptotic comets induced by serum withdrawal looked very different to the comets seen from heat shock or UV-C exposure, in that the apoptotic comet tail was wider and more diffuse, containing almost
all of the cell’s DNA (Figure 2.10). This confirmed that it would be possible to screen slides visually to determine the apoptotic fraction and see if treatment with 17β-estradiol would produce apoptotic cells.

Figure 2.10 - Appearance of apoptotic cell (left) and a cell with discrete DNA damage (right) following processing through the Comet assay. The apoptotic comet has the characteristic diffuse tail consisting of the majority of the DNA. (Magnification ×400).

On analysing the slides with the image analysis system, apoptotic comets were incorrectly assessed more often than not. The head of the comet is often so small that the low fluorescence is not measured and the true tail of the comet is assumed to be a comet head by the system. From the cells that are correctly assessed the Olive tail moments generally range from 40 to 70 μm, which is in good agreement with other cell lines described in the literature [Fairbairn et al., 1994; Olive et al., 1993].

2.6 Discussion

For our purposes, i.e. investigating the potential of 17β-estradiol, at low levels, to induce DNA damage in a population of cells, we required an assay that would prove to be at least as sensitive as alkaline elution.

The Comet assay was seen as a good option. We, like others, believe that 17β-estradiol is capable of inducing DNA damage in the form of strand breaks, AP sites and 7,8-dihydro-8-oxo-guanines (8-OHgua), which could be detected with the Comet assay. Numerous reports have placed the Comet assay higher than alkaline elution in terms of its sensitivity for detecting low levels of DNA damage [Leroy et al., 1996; Tice et al., 2000], and it offers a number of additional advantages. For example, the requirement for small numbers of cells per sample, which impacts positively on the amount of sample
required and thereby leads to lower costs. However, more important is the fact that the level of DNA damage in a single cell is measured. This allows the real impact of treatment within a heterogeneous cell population to be studied.

As a result of lysis there is a loss of more than 95% of the cellular proteins. The subsequent unwinding and electrophoresis stages at high pH facilitate denaturation, unwinding and expression of DNA single strand breaks and alkali-labile sites. Relaxed and broken DNA fragments migrate within the electrophoretic field at size-related rates to form a "comet tail."

The behaviour of the DNA can be explained by considering the organisation of DNA within the nucleus. When cells are permeabilised with detergent and nuclear proteins are extracted using high salt, the DNA remains within a residual nucleus-structure, the nucleoid. To a large degree the nucleoid maintains the supercoiling, but in the presence of strand breaks the higher structure relaxes and loops of DNA migrate through the agarose towards the anode during electrophoresis. Data suggest that the comet tail is made up of relaxed loops of DNA and that the number of loops in the tail (measured as %DNA in the tail) indicates the number of breaks [McKelvey-Martin et al., 1993].

It has been stated that it is useful, in terms of increased sensitivity, to have a small level of DNA migration in control samples [Tice et al., 2000]. We have paid heed to this advice and thus control cells in the Comet assay rarely exhibited a 0 μm tail moment in our hands. There is always some migration of the relaxed nucleoid in untreated samples.

In order to validate the Comet assay protocol we chose to use ultraviolet light (UV-C radiation, predominantly 254 nm). Ultraviolet light generates DNA base damage by many different mechanisms, including the production of oxygen radicals that lead to oxidative damage, and the generation of photoaddition products, particularly at adjacent pyrimidines, which readily form a cyclobutane pyrimidine dimer (CPD) across their C5-C6 double bonds.

The Comet assay detects the DNA single strand breaks that occur as a result of the attempted repair of the DNA damage induced by UV (so called excisable damage). For example, Endonuclease V initiates base-excision repair of thymine dimers commonly caused by UV damage to DNA. This enzyme is a combined DNA glycosylase/AP lyase, which forms a covalent Schiff's base intermediate that decomposes causing DNA scission at the phosphate between the pyrimidines of the
CPD. Nucleotide flipping then occurs, with the adenine in the complimentary strand being flipped to allow enzyme residues to be positioned for catalysis of the intrahelical CPD substrate. The intermediate strand breaks are thus detectable in the Comet assay.

In a population of cells not actively dividing, i.e. a 100% confluent flask, the level of repair was markedly lower than in the more active cells as indicated by the very low mean tail moment. In all three radiation dose levels the amount of damage detected was similar. It appears that the speed at which recognition and removal of damaged DNA bases occurs is seriously affected by metabolic activity. This would clearly have an impact upon studies involving detection of DNA damage visible only as a result of repair. In our case, however, we expected to induce DNA damage that would not require base excision repair to be detectable in the Comet assay, but nevertheless realised there would be benefits of ensuring cell populations in logarithmic growth phase were used.

Reproducibility from the UV studies was very good. UV acts much like a scatter-gun inducing damage randomly, not requiring, for example, transport into cells or metabolism, both of which we believe necessary for 17β-estradiol to induce DNA damage. As a result, although the reproducibility of the protocol appeared very high, we expected to see a great deal more variation when investigating the hormone. Due to the reproducibility in 70% confluent cells, UV-C was used as a positive control in subsequent studies.

The tail moment histograms produced from the samples treated with UV-C appeared well modelled by the $\chi^2$ distribution. The nature of UV-C radiation is such that it can be described as indiscriminate, being equally likely to damage one or another cell. With this in mind, it can be assumed that the histograms of tail moments will exhibit a high degree of unimodality, as seen from our data. As the level of damaged cells increased, the histograms began to resemble a more Guassian distribution, i.e. becoming more symmetrical. They were, however, still skewed, a factor that is taken into account when calculating the limits of error based on the $\chi^2$-fits.

Although one of the advantages offered by the Comet assay is the ability to investigate differences in susceptibility within cell populations, for statistical purposes it is sometimes necessary to condense the data into a summary measure. This is normally the arithmetic mean or the median value, but is either of these truly appropriate? The skewed nature of the tail moment distributions, away from that of a normal distribution
suggests that simply taking the arithmetic mean is unsatisfactory. The median value, however, may not fully represent the few very damaged cells in a population. By fitting the data to the $\chi^2$ distribution the parameter of the fit, the degree of freedom, is not only a summary measure but also indicates the skewness and allows the calculation of the limits of error. If its application is appropriate to a particular study it may prove more meaningful than either the normal mean and median. A major factor in this will be if, at high levels of DNA damage, the histograms are still unimodal.

MCF-7 breast cancer cells that have entered into the apoptotic pathway are clearly detectable, both by eye and following image analysis, in the Comet assay. Their hedgehog-like appearance is conspicuous and allows the slides to be initially screened to obtain an apoptotic fraction for each slide. Changes between treatments would be of significance and should be monitored.

It has been reported that once apoptosis is initiated the nucleosome fragmentation stage occurs very rapidly as cells with intermediate amounts of damage are not present at any time following induction [Olive et al., 1993]. This is in agreement with our findings, as demonstrated by a lack of tail moments below 20 $\mu$m.

The ability to reproducibly study low levels of DNA damage at the level of the single cell is clearly provided by the Comet assay. Chapter 3 presents the data resulting from studies investigating the DNA damaging capability of 17$\beta$-estradiol using this assay.
CHAPTER 3 - DO ESTROGENS, AT LOW LEVELS, INDUCE DNA DAMAGE?

The protocol introduced into the laboratory for the alkaline version of the Comet assay yields highly reproducible results with MCF-7 breast cancer cells irradiated with UV-C. This chapter describes the investigation into the DNA damaging potential of 17β-estradiol using the established Comet assay and a modified version of the protocol aimed at increasing its sensitivity.

As discussed previously, the genotoxic hypothesis involving estrogens has a relatively strong following [Yager and Liehr, 1996], largely based on reports such as that of Nutter et al. [1991]. Their data clearly demonstrate the ability of reactive metabolites of 17β-estradiol to induce DNA damage. Although the findings are significant, the damage was observed following treatment with high concentrations of 3,4-estrone-o-quinone, one of a number of potential metabolites. Tested concentrations of this estrogen quinone were in the range 10 - 500 µM, with acute toxicity being seen above 50 µM. As such high levels of the metabolite (>10 µM), are seen rarely, if ever, are these findings of any biological relevance? When considered with the other in vivo and in vitro data summarised in Chapter 2 it is unlikely that the findings of Nutter et al., are unimportant. They represent mechanistic studies that might explain the epidemiological data highlighting cumulative lifetime exposure to estrogens as a risk factor for certain types of cancers.

The production of 4-hydroxylated metabolites of 17β-estradiol can result in DNA damage. However, what this statement lacks is the supporting evidence that the metabolism of the parent hormone does in fact lead to the production of the appropriate metabolites at levels capable of inducing the DNA damage. In other words, it should be possible to detect DNA damage when cells are treated with the hormone itself. The aims, therefore, of this chapter are (i) to investigate whether 17β-estradiol, at a very low level, induces DNA damage, and (ii) probe the hypothesis that it is the quinone intermediates that cause the damage.

The cell culture and Comet assay protocols as described in the previous chapter required some small changes. They could not simply be applied to studies with 17β-estradiol. The nature of the test compound, as an endogenous hormone and potent
mitogen, leads to a number of potential problems if not considered prior to beginning the work. These are discussed in the next section.

3.1 Methodological considerations

Although not numerous, changes to the protocol will be set out separately in terms of cell culture and the Comet assay, and hopefully described in such a way that the reader might understand the thinking behind each decision.

Variations in growth media, serum concentrations and media supplements may have a significant effect upon the availability of estrogens to the cell and potentially the subsequent metabolism.

3.1.1 Cell culture

The composition of the growth medium used for routine culture of MCF-7 breast cancer cells varies to a certain degree from one laboratory to another. For instance, some laboratories use Dulbecco’s modified Eagle’s medium (DMEM), Roswell Park Memorial Institute 1640 medium (RPMI 1640), or like us α-MEM. When we first obtained our stock of MCF-7 cells they were growing in α-MEM and it was convenient to continue in that way.

Invariably, growth medium contains phenol red. The phenol red serves two main purposes; to act as an indicator of the medium’s quality in terms of pH; and to act as a buffer. Phenol red is in fact weakly estrogenic [Berthois et al., 1986], and therefore may complicate interpretation of results and eventual conclusions when studying estrogens. To avoid such problems our studies will use phenol red-free medium, which is buffered by other means, during the periods of treatment.

The growth medium is also supplemented with serum, usually fetal calf serum (FCS) at levels between 5 and 10% (v/v). Variations in the serum concentration used will have a profound effect upon the levels of free estrogens. Normally some 98% of estrogens are bound to serum proteins such as albumin and sex-hormone binding globulin (SHBG). Obviously, as we routinely supplement our medium with 5% (v/v) FCS, this will be kept constant for all the studies. However, quite apart from the
availability of free estrogens, there is another consideration. Fetal calf serum will contain estrogens in the form of endogenous hormones and growth hormones often given to cattle. The effect of these compounds upon study parameters is difficult to predict and therefore researchers often "strip" the serum of estrogens before use [Soto et al., 1995]. However, the growth hormones may persist after "stripping" and so for our studies we will supplement the growth medium with pooled human serum that has been "stripped" of its estrogens. In this way, we can be more confident that the concentration of 17β-estradiol used, although nominal, is not being increased by hormone already contained in the serum.

3.1.2 The Comet assay

Cell culture plasticware is significantly UV opaque, which explains why in the preceding chapter we needed to grow cells in petri dishes with lids. For irradiation purposes we removed the medium and placed the uncovered petri dish under the UV lamp for the required amount of time. In the case of the hormone studies, it was decided to grow the cells in 25 cm² cell culture flasks.

The other small change to the protocol is based on the type of damage we believe might be induced by the hormone. Unlike UV-photoproducts that require base excision repair to be initiated before the damage can be visualised, 17β-estradiol should result in forms of DNA damage that can be detected immediately. Thus, following the appropriate treatment period, the cells will be immediately processed via the Comet assay protocol.

3.2 Experimental procedure

Routine cell culture was carried out exactly as described in section 2.4.1. Experiments were repeated at least twice, often three times on separate occasions. To check analysis bias, on occasion, slides were coded prior to comet scoring by image analysis.
3.2.1 Removal of endogenous estrogens from human serum

This process referred to above as "stripping" was carried out on pooled human serum (National Blood Transfusion Service, London, U.K.) as previously described [Soto et al., 1995]. Briefly, activated charcoal was twice washed in double distilled (ultra high quality, UHQ) water. A suspension of 5% charcoal and 0.5% Dextran T70 (Amersham Pharmacia Biotech Europe GmbH, Freiberg, Germany) was prepared in a volume of UHQ water equivalent to that of the serum to be stripped, i.e. 250 mL. The suspension was pelleted by centrifugation (1000 × g, 10 minutes). The pellet was combined with the serum and mixed by rolling (10 rpm, room temperature, 1 hour). The mixture was then centrifuged for 20 minutes at 50000 × g and the charcoal-dextran stripped human serum (CDHus) filtered (0.2 μm, Nalgene, Merck Ltd. Dorset, U.K.) and stored at -20°C for up to six months.

A single batch (500 mL) of human serum was obtained from the Transfusion Service and was sufficient for the experimental work involving 17β-estradiol. Different batches of serum vary in terms of the fat and serum protein levels, both factors that could impact upon work of this kind, and so the same batch was used throughout.

3.2.2 Preparation of cells for treatment

In the descriptions that follow, the term "full α-MEM" refers to α-MEM with GLUTAMAX-I and phenol red supplemented with 5% (v/v) heat inactivated FCS. If the constituents of the growth medium differ at any point that will be made clear in the text.

A 25 cm² flask of 70% confluent MCF-7 cells was trypsinised and the cells resuspended in 20 mL full α-MEM, as described in section 2.4.2. Cells were enumerated and seeded in 25 cm² flasks at a density of between 2 - 4 × 10⁴ cells/mL, final volume of 5 mL, in full α-MEM. At the same time, for the purposes of the UV-C irradiated positive control, between 2 - 4 × 10⁴ cells/mL in a final volume of 3 mL full α-MEM were seeded in a petri dish with lid (3.5 cm diameter).

After 72 hours, the cells were washed with 5 mL HBSS and the medium replaced with stripped α-MEM, i.e. phenol red-free α-MEM supplemented with 10 mL L-glutamine (200 mM stock) and 5% (v/v) CDHus and with either 1% (v/v) ethanol or
test compound (see below). In the case of the positive control, a fresh 3 mL aliquot of full α-MEM was added following the HBSS wash stage.

3.2.3 Treatment of cells

A 1 mM stock solution of 17β-estradiol was made in absolute ethanol and stored in a glass container at -20°C. Appropriate dilutions were made up, also in absolute ethanol, and of the dilutions 50 μL was added to 5 mL stripped α-MEM before adding to the cells. The dilution was, therefore, a factor of 100. This meant that cells were exposed to less than 1% (v/v) ethanol, a dose that has previously been shown to have no detrimental effects on MCF-7 cells [Payne et al., 2000]. Final concentrations of 17β-estradiol in treated 25 cm² flasks ranged from 10 nM to 10 μM. Nominal concentrations were used.

One hour prior to the end of the dosing period, the positive control petri dish was irradiated with UV-C for 15 seconds (dose rate 7.6 J/m²/s) as described previously in section 2.4.2. Once the post-treatment incubation period had elapsed, this sample could be processed at the same time as the ethanol control and 17β-estradiol dosed samples.

3.2.4 Obtaining a single cell suspension and the Comet assay

A single cell suspension was obtained for the UV-C exposed sample as described in section 2.4.3. For the flasks, the volume of 0.25% trypsin-EDTA required was 660 μL and the cells were resuspended in 5 mL stripped α-MEM. In both cases, the cells were eventually suspended in PBS, at a known cell concentration. Cell culture medium containing estrogens was first disinfected and then stored until disposal by a regulated waste disposal company.

Processing of the cell suspensions in the Comet assay and their subsequent analysis was carried out exactly as described in sections 2.4.4 and 2.4.5.
3.3 Results

For each experiment at least fifty cells per slide were analysed, with duplicate slides for each treatment group. Comet assay results reported here represent pooled data from experiments repeated at least twice on separate occasions. The appropriateness of pooling data was confirmed using the Kruskal-Wallis one-way analysis of variance by ranks (Eq. (2.2)).

3.3.1 Controls

All experiments described in this chapter included both a negative solvent control {1% (v/v) ethanol} and a positive control (UV-C, 114 J/m²).

As part of the MCF-7 cell proliferation assay ongoing in the laboratory, MCF-7 cells are routinely incubated in medium containing 1% (v/v) ethanol for up to 5 days with no detrimental effect on cell viability or proliferation [Payne et al., 2000]. Nevertheless, we carried out a Comet assay upon untreated cells and cells incubated in 1% (v/v) ethanol for 24 hours (Figure 3.1). In both cases, levels of DNA damage were extremely low (χ²-fits to the data gave df values of 2.586 and 2.801, for untreated and ethanol controls, respectively), and no statistically significant difference existed (Wilcoxon-Mann-Whitney test, P > 0.05).

![Figure 3.1 - Tail moment histogram and χ² fit (blue line) of untreated and 1% (v/v) ethanol-treated MCF-7 cells. Treatment time was 24 hours and data are from two replicate experiments.](image)
The results indicate that there is no detrimental effect from the exposure to ethanol at the low final concentration of 1% (v/v) in the growth medium, and thus it was decided that an “untreated” control was superfluous. Clearly, it would be more valid to carry out comparisons of dosed samples to the solvent control.

The use of UV-C as the positive control was based on the knowledge that it produced reproducible levels of DNA damage detectable using the Comet assay (see Chapter 2). If there was little agreement between the results from the positive control sample and those seen normally, the experiment’s results would be considered even more critically than usual and the experiment would be repeated. Only one dose, normally 15 seconds (dose rate 7.6 J/m²/s), was included in a study. An example of the positive control histogram from one experiment is shown in Figure 3.2. In order to reduce the numbers of plots, they are not included in subsequent figures. Statistically, the data presented here were from experiments where the negative and positive controls were not different between studies (Kruskal-Wallis one-way analysis of variance by ranks, \( P > 0.05 \)).

![Figure 3.2](image)

**Figure 3.2 - Example of negative and positive control tail moment histograms and \( \chi^2 \) fits.** The degree of freedom (\( df \)) is calculated by the \( \chi^2 \) distribution (blue line).

### 3.3.2 Establishing an appropriate exposure time

Our initial studies were conducted using a relatively high concentration of 17\( \beta \)-estradiol (10 \( \mu \)M), over a range of exposure times (Figure 3.3). Exposure of MCF-7 cells to 10 \( \mu \)M 17\( \beta \)-estradiol for just 1 hour resulted in the induction of DNA damage as demonstrated by the small, but statistically significant, increase in tail moments (Wilcoxon-Mann-Whitney test, \( P < 0.05 \)). Treatment times above 1 hour resulted in increasing levels of DNA damage.
The histograms plotted from the tail moments exhibited a loss of unimodality after prolonged exposure to high concentrations of 17β-estradiol. Up to and including the 8 hour exposure, the histograms were asymmetrical but unimodal and the $\chi^2$-fits to the data were good. However, when the level of DNA damage was extremely varied as in the 24 hour exposure the $\chi^2$-fit appeared to fail.

![Figure 3.3 - DNA damage in MCF-7 cells exposed to 10 $\mu$M 17β-estradiol for various treatment times. DNA damage measured using the meter of Olive tail moment from the Comet assay. Histograms are fitted by a $\chi^2$ distribution (blue line). The comet image is a colourised image, digitised by the image analysis system. The level of fluorescence intensity is seen as different colours from blue (low) to yellow (high).](image)

Trypan blue exclusion indicated that at 10 $\mu$M, 17β-estradiol was deleterious to cell viability after 24 hours (Table 3.1). Thus the significant damage seen in this histogram may be indicative of toxicity at this dose and exposure time.
Table 3.1 - Effect of 10 µM 17β-estradiol on MCF-7 cell viability following increasing periods of exposure.

<table>
<thead>
<tr>
<th>Treatment time (h)</th>
<th>% viable a</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>96.1 ± 6.1</td>
</tr>
<tr>
<td>1</td>
<td>97.5 ± 4.9</td>
</tr>
<tr>
<td>2</td>
<td>94.2 ± 6.8</td>
</tr>
<tr>
<td>8</td>
<td>94.3 ± 7.3</td>
</tr>
<tr>
<td>24</td>
<td>77.9 ± 5.6</td>
</tr>
</tbody>
</table>

a measured by trypan blue exclusion, see section 2.4.3. n = 2.

3.3.3 Effect of different concentrations of 17β-estradiol

Having demonstrated that 17β-estradiol could induce DNA strand breaks and alkali-labile sites in MCF-7 cells when present at a high concentration, we investigated whether lower concentrations of the hormone would induce DNA damage.

Using an exposure time of 24 hours, MCF-7 cells were treated with 10, 100 and 1000 nM 17β-estradiol (Figure 3.4). There was no impact of these doses upon cell viability as measured by trypan blue exclusion.

Figure 3.4 - Impact of increasing doses of 17β-estradiol for 24 h upon tail moments in MCF-7 cells. $\chi^2$ Fits to the histograms are shown by the blue line and the parameter from the fit is df.
There is an obvious impact of the two higher doses on tail moment, but what is not immediately clear is that 10 nM 17β-estradiol induces a statistically significant increase in tail moment compared to controls (Wilcoxon-Mann-Whitney test, P < 0.05). Although the magnitude of the measured tail moments did not increase significantly, the proportion of cells with tail moments between 5 and 15 μm increased.

The χ²-fitting to the data of high levels of DNA damage (1000 nM 17β-estradiol), is again a cause for concern, clearly fitting the data poorly. At lower levels of damage the distribution appears to agree well with the χ²-fit.

### 3.3.4 Investigating the DNA damaging species

In an attempt to determine whether the DNA damaging species, in the case of 17β-estradiol, are free radicals or reactive oxygen species, dimethylsulfoxide (DMSO) was added to the medium. Details of the proposed pathway for the generation of the reactive species are outlined in section 2.2.1. DMSO acts as a scavenger of free radicals and reactive oxygen species, most notably the hydroxyl radical, however it has detrimental membrane effects and is therefore cytotoxic after prolonged exposure periods.

For these studies the test compound and DMSO (final concentration 0.05 % (v/v)), were added to the stripped α-MEM before the whole was aliquoted on to the attached cells. A DMSO-solvent sample was included in the studies to ensure that the DMSO was not causing any unwanted effects. The histograms in Figure 3.5 demonstrate that the presence of DMSO and ethanol in the growth medium did not have any detrimental effect upon the DNA of the MCF-7 cells. χ²-Fits to the data of the ethanol and ethanol-DMSO samples gave df values of 2.324 and 2.369, respectively, not significantly different. However, the presence of DMSO in samples with 17β-estradiol resulted in significant reductions in the magnitude of tail moments. At 1 μM 17β-estradiol (df = 11.956), the inclusion of DMSO reduced the χ²-fit parameter by over 50% (df = 4.708). Similar decreases in mean tail moments were observed for 100 nM 17β-estradiol, from df = 7.809 for the hormone alone to df = 3.154 when DMSO was present.
It is worth noting that even with DMSO present, 17β-estradiol, at both concentrations, induces statistically significant increases in DNA damage (Wilcoxon-Mann-Whitney test, P < 0.05).

![Figure 3.5](image)

**Figure 3.5 - Effect of radical scavenger upon 17β-estradiol induced DNA damage levels.** DMSO (0.05% (v/v)) was included in incubation mixtures. Blue lines are $\chi^2$-fits to the data, where $df$ is the degree of freedom from the fit.

### 3.3.5 Effects of hydroxylated metabolites of 17β-estradiol

The previous studies had indicated that 17β-estradiol was producing reactive species that were capable of inducing DNA damage and that this effect was attenuated in the presence of a radical scavenger. The genotoxic hypothesis *(Chapter 2)* suggests that it is the products of 17β-estradiol metabolism that produce the reactive species capable of creating an oxidative stress. If this is the case, it should be possible to detect DNA damage caused by the catechol metabolites, 2- and 4-hydroxy-17β-estradiol (2- and 4-OH E$_2$).
The two metabolites were very kindly synthesised and purified by Dr Michael Butterworth, as previously described [Butterworth et al., 1996; Butterworth et al., 1997], solubilised in absolute ethanol (final concentration of the catechol being 1 mM) and used in the Comet assay.

The appearance of DNA damage caused by 3,4-estrone-o-quinone was previously observed after one hour exposure [Nutter et al., 1991]. The catechol estrogens were unlikely to be as reactive as the o-quinone, but theoretically, the metabolites should induce DNA damage at a faster rate than the parent hormone. For this reason, a treatment period of 2 hours was used.

The results of exposing MCF-7 cells to 100 nM 17β-estradiol, 10 and 100 nM 2- and 4-OH E₂ and 1% (v/v) ethanol for 2 hours, as measured using the Comet assay, can be seen in Figure 3.6. At a concentration of 10 nM neither catechol estrogen produced an effect detectable above controls (df values for control, 10 nM 2-OH E₂ and 10 nM 4-OH E₂ were 2.406, 2.417, and 2.473, respectively). However, at 100 nM both 2-OH E₂

![Figure 3.6 - Tail moment histograms for MCF-7 cells exposed to 17β-estradiol, 2-hydroxyestradiol or 4-hydroxyestradiol. Cells were treated with the estrogen for a period of 2 hours. Blue lines are the χ²-fit to the data.](image-url)
and 4-OH E₂ produced significant increases in tail moments (\(df = 4.612\) and 6.721, respectively). Increases were statistically significant (Wilcoxon-Mann-Whitney test, \(P < 0.05\)).

### 3.3.6 Detecting 7,8-dihydro-8-oxo-guanine following estrogen treatment

The data thus far have demonstrated the ability of 17\(\beta\)-estradiol and its catechol metabolites to induce DNA single strand breaks and alkali labile sites in MCF-7 breast cancer cells.

The Comet assay can be modified using purified repair enzymes so that other forms of DNA lesion are detected, thus greatly increasing the assay’s sensitivity. The most frequently employed enzymes are Endo III, FPG and uvrA, uvrB and uvrC. Endonuclease III can be used to detect oxidised pyrimidines [Collins et al., 1993], formamidopyrimidine glycosylase (FPG) recognises 7,8-dihydro-8-oxo-guanines (8-OHgua) and other damaged purines [Dušinská and Collins, 1996], and uvrABC is used to detect bulky lesions and UV photoproducts [Collins et al., 1997].

7,8-Dihydro-8-oxo-guanine is a particularly abundant lesion, generated not only by oxidative stresses from the environment (e.g. chemical genotoxic compounds), but also as a by-product of normal cellular metabolism. 8-OHgua is highly mutagenic, yielding GC to TA transversions upon its replication by DNA polymerases [Boiteux and Radicella, 2000]. Interestingly, analysis of the sequence changes of \(p53\), a tumour suppressor gene that is very commonly mutated in cancers, showed that in many cancers there is a strong bias for the presence of GC to TA transversions [Naylor et al., 1987]. Based on this, we believed it important to investigate whether 17\(\beta\)-estradiol induced the generation of 8-OHgua with FPG.

Formamidopyrimidine glycosylase is an N-glycosylase with an associated \(\beta,\delta\)-lyase activity, which acts by catalysing the cleavage of both the 3’- and 5’-phosphodiester bonds of abasic sites in DNA, generating a single base gap limited by 3’ and 5’ phosphate ends. It is this strand break that is exploited in the Comet assay. Lesion recognition by FPG is relatively broad. Predominantly, formamidopyrimidines (derived from adenine or guanine), 8-OHgua and N7-methyl formamidopyrimidines are recognised, but FPG will also act upon some oxidised pyrimidine products, such as 5-hydroxyuracil and 5-hydroxycytosine.
To implement a repair enzyme step in the Comet assay, the treatment was carried out as previously described (3.2.2). However, following lysis, in a change from the normal protocol, the slides were washed three times with FPG buffer [0.1 M KCl, 0.5 mM Na₂EDTA, 40 mM N-(2-hydroxyethyl)-piperazine-N'-2-ethanesulfonic acid (HEPES) KOH, 0.2 mg/mL bovine serum albumin, pH 8.0]. FPG (PurOx-Cutter I, Cambio, Cambridge, U.K.) was diluted in 50 μL FPG buffer and was pipetted on to the surface of the gel and spread with a coverslip. Slides were then incubated for 1 hour in a humidified box at 37°C. The unwinding stage was skipped, instead progressing to electrophoresis, in the normal buffer.

For validation purposes, hydrogen peroxide (H₂O₂), which releases reactive oxygen species, was used. The medium in a 70% confluent 25 cm² flask was replaced with PBS containing H₂O₂ (50 μM). After 5 minutes on ice, the H₂O₂ solution was removed and replaced with fresh α-MEM and the cells were incubated for 1 hour at 37°C. Following the time for repair, the cells were trypsinised and put through the Comet assay protocol with the repair enzyme modification.

Following the period of repair, the level of DNA damage was relatively low (Figure 3.7), probably due to the fact that strand breaks are relatively rapidly repaired [Collins et al., 1995]. However, the FPG-sensitive lesions are only slowly repaired and it

![Figure 3.7 - The use of FPG to reveal base oxidation in MCF-7 cells treated with 50 μM H₂O₂. Treatment (5 minutes) was followed by an hour incubation for repair before running the Comet assay. 2 U and 5 U FPG are 2 and 5 units FPG, where one unit is defined as cleavage at the rate of 100 fmol/h.](image)
was these that became evident following FPG treatment. The sensitivity offered by using 2 units of FPG, where one unit is defined as cleavage at the rate of 100 fmol/h, compared to that of the recommended 5 units was sufficiently high to allow the use of 2 units FPG per slide in later experiments, which had a very beneficial effect on running costs. The effect of hydrogen peroxide on MCF-7 cells was such that every cell analysed had a degree of DNA damage in the form of 8-OHgua or alike, demonstrated by the fact that no comets were observed with tail moments in the range 0 to 3 μm.

The application of the FPG modification step to the damage induced by 17β-estradiol and its catechol metabolites is shown in Figure 3.8 and the $\chi^2$-fit parameters are summarised in Table 3.2.

![Figure 3.8](image)

Figure 3.8 - 8-OHgua levels in MCF-7 cells treated with 100 nM 17β-estradiol, 2- or 4-OH E2 for a duration of 2 hours. Tail moment histograms of cells incubated with FPG buffer alone (orange outlined bars), and cells incubated with FPG (grey bars) are fitted with the $\chi^2$-distribution to the data (orange and blue curves, respectively).

As mentioned earlier, cells tend to have low background levels of 8-OHgua due to cellular metabolism [Collins et al., 1996], and this is evident in the controls treated with FPG (Table 3.2). The levels are low, but statistically significant (Wilcoxon-Mann-Whitney test, P < 0.05). Comparison of the FPG buffer control histogram with that of controls in previous studies (for example, Figure 3.1), shows that the buffer may be increasing the levels of DNA damage observed. It is likely that the hour incubation (37°C) of the slides in FPG buffer, which replaces the 20 minute unwinding stage before
electrophoresis, is allowing more alkali labile sites to be expressed as breaks. Also, effectively extending the period in which the nucleoid is unwinding may lead to more DNA being able to migrate in the gel, although there is no significant increase in the level of damage.

Treatment of estrogen dosed cells with FPG led to a significant increase in the level of DNA damage detected in the Comet assay (Wilcoxon-Mann-Whitney test, \( P < 0.05 \)). In the case of 17β-estradiol, FPG treatment resulted in a 73% increase in DNA damage observed. For the catechol metabolites, the levels of 8-OHgua were even higher, leading to almost a six-fold and a four-fold increase in detected damage for 2-OH \( \text{E}_2 \) and 4-OH \( \text{E}_2 \), respectively (Table 3.2).

<table>
<thead>
<tr>
<th>Treatment (^a)</th>
<th>( df ) value (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control + FPG buffer</td>
<td>4.853</td>
</tr>
<tr>
<td>Control + FPG</td>
<td>5.320</td>
</tr>
<tr>
<td>100 nM ( \text{E}_2 ) + FPG buffer</td>
<td>8.996 (^c)</td>
</tr>
<tr>
<td>100 nM ( \text{E}_2 ) + FPG</td>
<td>15.525 (^c,d)</td>
</tr>
<tr>
<td>100 nM 2-OH ( \text{E}_2 ) + FPG buffer</td>
<td>4.643</td>
</tr>
<tr>
<td>100 nM 2-OH ( \text{E}_2 ) + FPG</td>
<td>27.780 (^c,d)</td>
</tr>
<tr>
<td>100 nM 4-OH ( \text{E}_2 ) + FPG buffer</td>
<td>6.715 (^c)</td>
</tr>
<tr>
<td>100 nM 4-OH ( \text{E}_2 ) + FPG</td>
<td>24.348 (^c,d)</td>
</tr>
</tbody>
</table>

\(^a\) treatment 2 h; FPG incubation 1 h
\(^b\) \( df \) values represent the parameter of the \( \chi^2 \)-fit to the data (Figure 3.8)
\(^c\) significant DNA damage compared to control + FPG buffer given by the Wilcoxon-Mann-Whitney test, \( P < 0.05 \)
\(^d\) significant DNA damage compared to equivalent treatments with FPG buffer given by Wilcoxon-Mann-Whitney test, \( P < 0.05 \)

The \( \chi^2 \)-distribution fitted to the data was good in all cases. An element of skewness was evident in all the samples, even the FPG treated catechol metabolite samples, where the tail moment histograms are tending to normal in appearance (Figure 3.9).
3.3.7 Estrogen induced DNA damage and the estrogen receptor

The MCF-7 breast cancer cells are estrogen receptor positive. In terms of ERα, activation of the receptor by the ligand will eventually lead to cellular proliferation, this being the basis of the MCF-7 cell proliferation assay. Is it therefore conceivable that the increase in tail moments of MCF-7 cells exposed to estrogens is not due to DNA lesions but is, in fact, artefactual and instead resulting from DNA replication?

There is evidence that the mitogenic effect of 17β-estradiol in vivo can be detected relatively shortly after administration, for instance cGMP content increases within 2 hours [Galand et al., 1987]. However, the proportion of MCF-7 cells in S phase was increased by approximately 10% over control values 24 hour after dosing [Bezwoda and Meyer, 1990]. The expert panel meeting at the International Workshop on Genotoxicity Test Procedures gave no preference to the use of proliferating versus non-proliferating cells in the Comet assay [Tice et al., 2000].

To confirm the relevance of our previous findings, we carried out a study using a breast cancer cell line that is ERα-negative and therefore does not proliferate in
response to 17β-estradiol. If the results from experiments incubating ER-negative cells with estrogens concurred with our results with MCF-7 cells, it would indicate that the damage was not mediated via the estrogen receptor. The MDA-MB-231 cell line, a kind gift from Dr Michael Johnson (Lombardi Cancer Center, Washington DC, USA), was maintained in Dulbecco’s MEM with Glutamax-I and phenol red supplemented with 5% (v/v) FCS.

The protocol for the Comet assay was as previously described (section 3.2.2) with one change; the DMEM with phenol red was replaced with phenol red-free DMEM with 10 mL L-glutamine (200 mM stock) and 5% (v/v) CDHus, instead of the α-MEM equivalent.

Following exposure of MDA-MB-231 cells to 100 nM 17β-estradiol, 100 nM 2- or 4-hydroxy-17β-estradiol for 2 hours, there was an increase in the level of DNA lesions as detected in the Comet assay (Figure 3.10).

![Figure 3.10 - Effect on tail moment of MDA-MB-231 cell treatment with 17β-estradiol or its catechol metabolites. Treatment duration 2 h.](image)

All estrogen-induced increases in DNA damage were statistically significant compared to controls (Wilcoxon-Mann-Whitney test, P < 0.05). Compared to the equivalent study in MCF-7 cells, the level of damage as indicated by the $\chi^2$ distribution fitted to the data was not significantly different (Table 3.3). In fact, the agreement between the ER-positive and the ER-negative cell lines was somewhat extraordinary.
Table 3.3 - Cell line comparison of DNA damage induced by various estrogens at 100 nM.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MCF-7</th>
<th>MDA-MB-231</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.406</td>
<td>2.905</td>
</tr>
<tr>
<td>100 nM E2</td>
<td>4.740</td>
<td>4.082</td>
</tr>
<tr>
<td>100 nM 2-OH E2</td>
<td>4.612</td>
<td>4.424</td>
</tr>
<tr>
<td>100 nM 4-OH E2</td>
<td>6.721</td>
<td>6.245</td>
</tr>
</tbody>
</table>

* values are degree of freedom obtained from the $\chi^2$-fit to the data

\[ a \] comparison of same treatment in different cell line using Wilcoxon-Mann-Whitney test, ns - not significant, $\alpha = 0.05$.

3.4 Discussion

Our data add support to the hypothesis that 17β-estradiol is a weak mutagen capable of inducing genetic lesions. Exposure of MCF-7 breast cancer cells to 17β-estradiol resulted in the formation of not only DNA single strand breaks (SSB) and alkali labile sites (ALS) but also the highly mutagenic lesion, 7,8-dihydro-8-oxo-guanine (8-OHgua).

Using the Comet assay, a detectable increase in DNA SSB and ALS was seen following 24 hour exposure of MCF-7 cells to concentrations of 17β-estradiol as low as 10 nM. DNA lesions at this concentration have not been reported previously, probably a direct result of the increased sensitivity offered by the Comet assay over other DNA damage detection methods [Leroy et al., 1996]. It is unlikely that this small increase in damage would have been detected had a tail moment histogram based on individual cells not been constructed. Pooling cellular DNA, as in the case of alkaline elution, may mask small effects, such as this. However, it must be noted that even this level of the hormone, although low, is still approximately three orders of magnitude higher than normal female physiological concentrations. The implications of this, in terms of the relevance of these findings to the in vivo human situation are further discussed in Chapter 7.

More in-depth investigations showed that the DNA damaging effects of 17β-estradiol could be markedly reduced by the presence of a free radical scavenger, in this case dimethylsulfoxide. Mean tail moments, as given by the parameter (df) of the $\chi^2$-fit to the data, were more than halved in samples with DMSO. These data clearly indicate a
strong involvement of free radicals or reactive oxygen species in the mechanism by which 17β-estradiol induces comet formation. The inability of DMSO to completely eradicate increases in the level of DNA damage in treated samples compared to controls, points to the presence of DNA lesions that are not induced by reactive oxygen species. A plausible explanation is forthcoming when the ability of metabolites of 4-hydroxylated estrogen, namely estrogen-3,4-quinones, to form depurinating adducts is remembered. The apurinic sites being detectable in the alkaline version of the Comet assay as they are alkali labile.

The proposed metabolic activation pathway for 17β-estradiol (Figure 2.2), suggests that it is the catechol estrogen metabolites that, following conversion to the catechol estrogen quinones, undergo redox cycling and eventually result in the formation of reactive oxygen species. By removing the initial metabolic step, i.e. the conversion of 17β-estradiol to the catechol estrogens, and exposing the cells to 2- or 4-hydroxyestradiol directly, we expected to see a substantial change in the kinetics and extent of DNA damage formation. This was, however, not the case. Following a 2 hour treatment with 2-OH E₂ the mean tail moment was slightly lower than that of the parent hormone at the same concentration. The other tested catechol estrogen, 4-OH E₂, did induce statistically higher levels of DNA SSB and ALS compared to 17β-estradiol, the difference being modest.

A potential explanation for the lack of an increased level of DNA damage following treatment with the catechol estrogens was provided by the inclusion of the formamidopyrimidine glycosylase (FPG) digestion step in the assay protocol. In all three instances, i.e. treatment with the parent hormone and the catechol estrogens, there was a marked shift of the tail moment histograms to the right following FPG treatment, indicating elevated levels of damage. This is indicative of the presence of 8-OHgua in the DNA of treated MCF-7 cells. The magnitude of the shift observed in the cases of the metabolites was extremely high. The predominant form of DNA damage induced by 2- and 4-OH E₂ was clearly oxidised bases and this explains why the unmodified protocol failed to detect a marked increase in the level of DNA SSB and ALS. What these findings cannot explain is why the catechol estrogens produce 8-OHgua in apparent preference to DNA SSB or ALS. More detailed studies will be required in order to discover the answer to this.
There is a clear implication of reactive oxygen species from the FPG studies, in agreement with the proposed metabolic activation pathway. Hydroxyl radicals, potential products of superoxide radical metabolism, can produce DNA damage [Liehr and Roy, 1990; Han and Liehr, 1994b; Nutter et al., 1991; 1994], predominant forms of which are 8-OHgua and the adenine equivalent, 7,8-dihydro-8-oxo-adenine. Both hydroxylated base lesions have been found to be abundant in the mammary DNA of breast cancer patients [Malins et al., 1993], and prostate DNA from patients with cancer of the prostate [Malins et al., 1997].

The controversy surrounding which of the catechol estrogens is more genotoxic is, however, not resolved in any way by these data. Although the levels of DNA fragmentation are significant, the differences between the tail moment histograms for 2- and 4-OH E\(_2\) are minor.

Estradiol induces kidney tumours in Syrian hamsters, as does 4-hydroxyestradiol but not 2-hydroxyestradiol [Liehr et al., 1986]. As a result, 2-hydroxylated estrogens are widely designated as “good estrogens” and considered as improbable carcinogenic metabolites in humans [Schneider et al., 1984]. Newbold and Liehr [2000], recently reported “the hamster kidney is a mechanistic model and not an organ model for the genesis of human hormone-associated cancers, as, for instance, in the breast or uterus.” They went on to demonstrate their point by describing a degree of 2-OH E\(_2\)-induced uterine adenocarcinoma in CD-1 mice. The most likely encountered catechol estrogen in human breast tissue will be 4-OH E\(_2\), as 4-hydroxylation of estrogens is the predominant route, due to the specific estrogen 4-hydroxylase that is detectable in tumours and normal breast tissue [Liehr and Ricci, 1996]. Taken together, these considerations and our data suggest that the DNA damage in MCF-7 cells following 17\(\beta\)-estradiol treatment is due to metabolism via the 4-hydroxylated estrogen and the resulting redox cycling generated by enzymatic reduction of the quinone to semi-quinone and auto-oxidation back to quinone. What our findings with 2-OH E\(_2\) demonstrate is that significant levels of oxidised bases would result in MCF-7 human breast cancer cells, were 17\(\beta\)-estradiol to be metabolised to the 2-hydroxylated estrogen.

Similar levels of DNA damage were seen in the ER\(\alpha\)-negative MDA-MB-231 cell line following estrogen treatment. This consistency with the MCF-7 data, in the absence of an estrogen-responsive proliferative pathway, leads us to conclude that the
results are consistent with an increase in genotoxicity and not due to an increased rate of cell division.

The application of the Comet assay to the question of detecting low levels of DNA damage in cell populations exposed to estrogens is clearly appropriate. In conjunction with repair enzymes, such as FPG, the sensitivity of the technique surpasses that of other methods [Collins et al., 1996; Leroy et al., 1996], and the ability to visualise the extent of DNA fragmentation in the individual cell enables changes within populations to be dissected in more detail.

In Chapters 2 and 3 we utilised a method of analysing the tail moment data first reported by Bauer et al. [1998], namely the fitting of the $\chi^2$-distribution to tail moment histograms. Their initial work was carried out using UV-A irradiation as the DNA damaging agent, and as with our validation studies using UV-C, there was excellent agreement between the distribution of the tail moments and the fitted $\chi^2$-distribution. However, the distribution of DNA damage from agents such as UV tends to be very uniform in heterogeneous cell populations. Chemical genotoxic agents, however, are highly influenced by a cell's sensitivity and metabolic profile and within a heterogeneous cell population responses may be quite disparate. In contrast to UV-induced damage, with such a chemical agent, we may not see the tail moment population distributions tending to a Gaussian shape as the levels of damage increase.

As expected, for low levels of DNA damage induced by 17$\beta$-estradiol, the $\chi^2$-distribution modelled the data well, but for higher hormone concentrations confidence in the fit to the data decreased (Figures 3.4 and 3.5). There is the suggestion that the DNA damage seen following 24 hour treatment with 10 $\mu$M 17$\beta$-estradiol may have been affected by the onset of necrosis. At this dose there was a significant drop in the proportion of viable cells, and there is also evidence that over longer exposure periods, 1 $\mu$M 17$\beta$-estradiol is cytotoxic [Soto et al., 1995]. Apart from these two concentrations, the $\chi^2$-distribution appeared appropriate for the tail moment histograms obtained in these studies. Additionally, as a result of the fitting procedure, a single parameter is obtained that describes the histogram and which allows the calculation of limits of error. This represents a useful addition to the data analysis techniques and due to the often skewed nature of the distributions may be more appropriate than the classical statistical methods based on ranks and arithmetic means.
In summary, in human breast cancer cell lines, exposure to 17β-estradiol or the catechol estrogen metabolites, at 100 nM, for periods as short as 2 hours, results in the induction of DNA lesions. Furthermore, the predominant lesions are oxidised bases (8-OHgua) that are slowly repaired and in rapidly proliferating cells, such as those of the breast, can easily increase critical mutagenic events [Cavalieri et al., 1997].
The previous chapter described the DNA-damaging properties of 17β-estradiol, and its 2- and 4-hydroxylated metabolites. This was demonstrated by an increased formation of DNA single strand breaks, alkali labile sites and 7,8-dihydro-8-oxo-guanine moieties in treated human breast cancer cell lines. The epidemiological studies in humans clearly identify estrogen as a carcinogen, but only a weak carcinogen [IARC, 1999], adding approximately 3% to the breast cancer risk for every year of exposure to estrogen [Beral et al., 1997]. This is to be expected given estradiol’s role as an endogenous hormone at low picomolar levels and considering that a strong carcinogenicity would have provided an evolutionary disadvantage to humans and many other species [Liehr, 2000].

If it is the case that only a small proportion of the reports of increases in detrimental health effects in Man and wildlife is accounted for by the weak carcinogenicity of endogenous estrogens, what other factors could be involved?

In wildlife, exposure to high levels of certain chemicals, namely those that mimic steroidal estrogens, such as halogenated pesticides and polychlorinated biphenyls (PCBs), has been shown to result in detrimental health effects. However, this link has only been conclusively proven where high level exposure to particular xenoestrogens (XEs) has occurred, such as the Tower Chemical Company spill that contaminated Lake Apopka [Semenza et al., 1997].

Fortunately, instances of exposure to such high levels of XEs are rare. Even so, some used the limited observations to formulate hypotheses that propose a significant role to environmentally persistent XEs in the detrimental effects reported not only in wildlife [Colborn and Clements, 1992], but also in Man [Sharpe and Skakkebaek, 1993; Davis et al., 1998]. As a result, research continues to screen the wide variety of structural classes of natural and industrial compounds to assess their estrogenicity [Blair et al., 2000]. Most significant are organochlorine pesticides (e.g. o,p’-DDT, methoxychlor, toxaphene, chlordane, endosulphan, dieldrin, and kepone), other organochlorine compounds (PCBs and hydroxylated PCBs), phenolics (bisphenol A, nonylphenol, and octylphenol) and phthalates [Korach et al., 1988; Soto et al., 1991; 1994; 1995; Krishnan et al., 1993; White et al., 1994;
Jobling et al., 1995]. These screening exercises have served an important cause, that of establishing the sheer number of xenoestrogens to which we are potentially exposed.

Whilst screening continued, epidemiologists began investigating whether an association existed between exposure to XEs, determined by serum or adipose tissue levels, and breast cancer risk. The conclusions from the numerous studies are, as discussed previously (1.2.3), conflicting. Although the hypotheses are compelling, the data are far from convincing.

What has come of all the research in the field, to date, other than a sense of the sheer number and environmental persistence of the XEs we are potentially exposed to, is that the vast majority of XEs are considerably less potent than the steroidal estrogens and only present in human tissues at low levels. It is the latter two points that lead some to argue, “it is unlikely that the industrial estrogens play a role in breast cancer in women and reproductive disorders in males” [Safe, 1995b]. Safe's assertion that XEs are too weak and not present at high enough concentrations to represent "real risk" is based upon single agent estrogenicity data that indicate the need for implausibly high concentrations to elicit effects. He discounts as unimportant, in our opinion naively, the fact that, although present individually at low concentrations, their number is in the hundreds [Blair et al., 2000]. By considering each of these XEs on an individual basis, Safe's assertion that the public should be reassured by the epidemiology data [Safe, 1997], is unlikely to be experimentally dismissed.

At any one time, exposure is not to just one XE but to a whole multitude of estrogenic agents [Gillesby and Zacharewski, 1998]. It is no longer appropriate to consider agents individually [Payne et al., 2000; 2001; Silva et al., 2001]. It is conceivable that the effects of one XE may be altered, enhanced or diminished, in the presence of another XE.

The assessment of mixtures of estrogenic compounds is, therefore, a matter that governmental agencies now regard as a major research priority [Reiter et al., 1998]. It is evident from the xenoestrogen literature, however, that the theoretical basis of mixture analysis is often poorly understood and as a result, studies tend to be ill-conceived [reviewed by Yang, 1998; Kortenkamp and Altenburger, 1998, 1999; Borgert et al., 2001].

In order to correctly assess combination effects of chemicals, it is crucial to formulate a hypothesis about the expected effect of the mixture. This hypothesis (the null-hypothesis) provides the necessary basis for evaluating mixture effects in terms of synergism or antagonism. Evaluations of the combined effects of agents, therefore, are
highly reliant upon the method used to estimate the expected effect of a mixture. An appropriate reference point for such analyses is the expectation that joint effects are the result of purely additive interactions of all mixture components [Berenbaum, 1985; Borgert et al., 2001]. If the observed combination effects exceed the expected additive effects, there is synergism; if the observed response falls short of expectation, there is antagonism. This task of defining the expected additive effect of a mixture, however, is not a trivial one and is discussed in more detail below (section 4.1).

It is perceived that to result in a real biological impact, the fact that the XEs possess low potency and are present at low tissue and environmental levels, the interaction between XEs needs to demonstrate synergism [Bergeron et al., 1994; Arnold et al., 1997]. The majority of the data from well-conducted binary, ternary and even quaternary XE mixture investigations have revealed only additive mixture effects [Bergeron et al., 1999; Payne et al., 2000]. The apparent all-consuming desire to seek out synergisms between xenoestrogens is, in our opinion, very unhelpful. By not detecting synergisms many presume it to indicate "no risk," implying that purely additive combination effects between XEs are unimportant and not relevant to the negative health outcomes observed. So far, this complacency is not based on empirical data.

And so, in our eyes, two questions present themselves. Firstly, are there mixture effects with combinations of xenoestrogens at individually low-effect concentrations? And if so, what are the consequences of the XEs' joint actions when one remembers that, to be of biological significance, they must impact upon the already potent effects of the endogenous hormone, 17β-estradiol? In order to answer these questions there are a number of theoretical issues that need to be addressed. It is important to have these issues clarified prior to beginning studies, as they may have considerable impact upon experimental design. The significant areas are, (i) how combination effects are assessed; (ii) how low-effect concentrations are established, and; (iii) how the choice of assay is affected by (i) and (ii).

4.1 Expectation and concepts to define it

The number of XEs identified in the environment is already in the hundreds and this number continues to rise as more chemicals are assessed for estrogenicity. Exposure, however, is not universally the same. For example, let us consider two individuals, one
from an industrialised country of the West and the other from a developing nation. The ubiquitous nature of XEs means that both individuals will be exposed, but there will be considerable differences not only in the number but also concentrations of XEs that each is exposed to. The challenge, therefore, is to establish the effects of exposure to these different mixtures. Clearly, the number of permutations of mixture components makes attempting to assess experimentally all possible combinations highly impractical.

An alternative approach involves utilising information concerning the relationship between dose and response for each individual XE. Using the single agent information, pharmacologically sound, mathematical concepts enable the derivation of predicted mixture effects, i.e. define the expected effects for a mixture of known composition. Three main concepts for the prediction of (expected) additive combination effects on the basis of data about the potency of individual mixture components are commonly encountered in the literature [reviewed in Berenbaum. 1989; Kortenkamp and Altenburger, 1998, 1999; Borgert et al., 2001], namely, effect summation, concentration addition, and independent action. These empirical concepts promise the ability to estimate the effect of a mixture of agents based solely on information of the single agents and the ratio at which the components are combined. The choice of model to define the expected mixture effect is a key aspect of mixture analysis and is discussed below. By applying the appropriate model, an explicit definition of the expected (additive) mixture effects can be made. Valid conclusions as to the nature of the mixture effect will be ensured by comparing experimental mixture data with the additivity reference.

Before entering into the discussion of concepts, it may be helpful to briefly describe the type of mixtures that this thesis concentrates upon. A combination of agents all of which produce the effect under observation is termed homergic. For example, this is a mixture of XEs that all produce increases in uterine wet weight in the rodent uterotrophic assay. When a mixture is composed of agents that do not all produce the effect chosen for analysis, it is termed heterergic. An example is the antitumour effect of methotrexate and folinic acid, which is only attributable to the former but the presence of the folinic acid potentiates its effect. It is not possible to predict the combination effects of heterergic mixtures from features of the single agents' dose-response relationships. Heterergic mixtures are beyond the scope of this thesis, and will not feature in the subsequent discussions or investigations.
4.1.1 Effect summation

Effect summation (ES) is a widely used method of calculating expected mixture effects, also in the xenoestrogen field [Soto et al., 1994; Arnold et al., 1997]. ES assumes that the combined additive effect of a mixture should always be equal to the arithmetic sum of the effects of its components. Deviation from this expectation is then classified as synergism or antagonism. ES is intuitively appealing, probably as a result of the simple experimental design. Both the single agents and the mixture are tested at one dose level, and the effect produced by each agent individually is summed before comparison with the response yielded by the mixture consisting of the combined doses. It is frequently overlooked that this concept can only be applied when dealing with agents that exhibit linear dose-response relationships [Berenbaum, 1985]. Sigmoidal dose-response curves, which are seen in the estrogen field, will produce very unreliable predictions (Figure 4.1). Its application, in this thesis, serves only to reiterate this fact. It is inappropriate for mixtures of agents with sigmoidal concentration-response curves. These shortcomings are highlighted further in Chapter 6.

Figure 4.1 - A sham experiment demonstrating the inappropriateness of effect summation for mixtures of agents with sigmoidal dose-response curves. Compound C exhibits a sigmoidal dose-response curve, at 0.03 μM it causes an arbitrary effect of 0.23 units (solid black line). Effect summation would predict that two aliquots, each of 0.03 μM compound C would result in an effect equal to 0.23+0.23 i.e. 0.46 units (dashed line). In fact, 0.06 μM compound C results in an effect of 0.87 units (solid red line). Such a result would lead to the illogical conclusion that compound C synergises with itself!
4.1.2 Concentration addition

The concept of Concentration addition (CA) [Frei, 1913; Loewe and Muischnek, 1926], assumes that the components of a mixture act in a similar way and have a common site of action. The concentrations of the single agents in the mixture are expressed as fractions of equi-effective individual concentrations, and these quotients are termed toxic units. The effect of a mixture is expected to remain constant if the sum of the toxic units remain constant, i.e. a component of the mixture may be replaced in part or in total by an equal fraction of an equi-effective concentration of another and the mixture effect will be the same. Figure 4.2 demonstrates the basis of concentration addition using a useful graphical application of CA, i.e. the method of isoboles (iso-effect curves).

Figure 4.2 - Derivation of an isobole for two hypothetical, homergic drugs and testing of the interaction. A: The dose-response curves for two drugs are shown (blue curves). An additivity response surface can be drawn for mixtures of the two drugs. This is, in effect, made up of numerous lines connecting equi-effective mixture concentrations, eg. effects of 25, 50 and 75% control (mauve lines). Graph A is viewed from above giving graph B: where the equi-effective concentrations are shown for effects of 25, 50 and 75% control (mauve lines). Experimental testing of two mixture ratios of the drugs was carried out for various concentrations, and concentrations of the mixtures that produced effects of 25% control were determined. Knowledge of the mixture ratios makes it possible to plot these points on the isobole (grey circles), and an isobole (black line), can be drawn through these points and the equi-effective concentrations of the single agents. As the observed isobole is concave upwards, i.e. below the additivity isobole, the combination is synergistic.
Although very useful for graphically representing the analysis of combination effects, isoboles can only be used in studies investigating binary (Figure 4.2), or tertiary mixtures. It is, clearly, not possible to represent more than three-dimensions graphically, for example, a four component mixture isobole would require four axes to draw the response surface isobole.

It is generally agreed that CA is a suitable and valid concept for the prediction of mixture effects of similarly acting agents [EPA, 1986; EIFAC, 1987]. Its applicability to mixtures of agents that exhibit different modes of action is embroiled in controversy [Berenbaum, 1985], and is vehemently denied by some experts [Chou and Rideout, 1991; Pöch, 1991]. The equations involved in deriving CA predictions are featured in Chapter 5, where this concept is applied to predict the effects of various binary mixtures.

4.1.3 Independent action

Independent action (IA), in contrast to CA, assumes that compounds act via different sites and with different modes of action but result in the same measurable effect [Bliss, 1939]. Thus, the effects of the individual mixture constituents are independent in a probabilistic sense, i.e. the relative effect of a toxicant remains unchanged in the presence of another chemical [Faust et al., 2000]. As such, combination effects cannot occur if the components of the mixture are present at concentrations below their individual effect thresholds. Doubts do exist as to the general applicability of IA to integral endpoints such as cell death or cell division and endpoints at higher physiological levels [Greco et al., 1995], or under conditions of chronic exposure [EIFAC, 1987]. Again, as with CA, calculations of the expected effect of a combination using IA are presented in Chapter 5.

4.1.4 Implications for choice of assay - Part one

Conceptually, each model operates quite differently, but a prerequisite for all is a well-characterised concentration-response relationship for each component of the mixture and precise knowledge of the mixture composition. Without robust description of the single agents, the predictive power of each concept will be compromised, which may
lead to erroneous conclusions [Borgert et al., 2001]. As described by Backhaus et al. [2000], a priority is necessarily low effect concentrations:

Independent action relies on known effects of the individual substances for predicting the overall effect of a mixture. As the predicted mixture effect is always larger than the effects of the mixture components alone, this means that, in the case of multiple mixtures, rather low effects of the single compounds are to be described.

It is clear, therefore, that in order to evaluate combination effects accurately, it is vital to place a high premium on detailed concentration-response analyses of both the individual agents and, subsequently, the mixtures. Thus, large number of data are needed, and this carries both ethical and cost implications which point to the use of in vitro assay systems that are amenable to high through-put screening.

4.2 The estimation of low-effect concentrations

From the earlier descriptions of concentration addition and independent action, it should be apparent that differences exist as to the significance of low-effect concentrations. The assumptions that underlie CA imply that mixture constituents are expected to contribute to the effect of the mixture. This is the case for all concentrations, no matter how low, i.e. for each individual component there is no threshold concentration other than zero. If, however, the effect of a mixture is predicted using IA, the situation is quite different. To contribute to the overall effect of the mixture, an individual constituent must cause an effect greater than “zero.” In this instance, “zero” is more often described by the control value. For the predictive models, therefore, it is important to be able to accurately estimate low-effect concentrations, especially so that a zero effect level can be reliably approximated for IA.

It has been mentioned earlier that, typically, XEs are detected in the environment and biological media at very low concentrations. These levels are below that which will elicit statistically significant effects in laboratory assays, i.e. they are below or at their No Observed Effect Concentrations (NOECs). This fact appears to form the foundation for the belief that due to their apparent individual “ineffectivity” there is “no risk.” When considering mixtures, it is assumed that combination effects according to the concept of independent action cannot occur if concentrations of mixture components do not exceed individual NOEC values [Grimme et al., 1996]. In this instance, the NOEC
value has been used as the approximation of the real zero effect level or No Effect Concentration (NEC), however it is a poor estimate of this actual concentration [Chapman et al., 1996].

4.2.1 Inappropriateness of NOEC values

The regulatory use of NOECs, defined as that largest tested concentration of an agent for which the chosen end-point is not significantly different in the exposed group and the control group, has recently been subjected to much criticism [Chapman et al., 1996; Moore and Caux, 1997]. Criticism stems from the methodology of deriving NOEC values, which is considered very limiting. For example, the NOEC value is restricted to one of the experimental dose levels, does not fully utilize the dose-response information and has no error terms.

The emphasis of establishing NOEC values is not on risk estimation but rather on hypothesis testing. The null-hypothesis being that the effects of treatments are not different from that of the effect of the controls, and if this hypothesis is rejected a post-hoc multiple comparison test (e.g. Dunnett’s or Tukey’s test), is used to establish the NOEC. Moore and Caux [1997], whilst exploring a regression-based approach for estimating low toxic effects (discussed below, 4.2.2) demonstrated that, in fact, NOEC values derived from toxicity tests represent reductions from the control response of between 10 and 30%. Since NOEC values are inappropriate for the calculation of expected mixture effects according to IA, an alternative is needed.

4.2.2 The regression-based approach to estimate low-effects

A more reliable method for estimating low effect concentrations has been proposed [Crump, 1984; 1995], namely the regression-based approach. This approach involves, firstly, estimating the concentration-response relationship by fitting a regression-based model to the bioassay data. The second step is interpolating or extrapolating to the effect level of interest, e.g. 10% effect concentration (EC10), a so-called point estimate. ECx point estimates are also referred to as benchmark doses; the doses that correspond to specified levels of increased response [Crump, 1995]. In this way, an alternative to the
NOEC “threshold” can be derived. Confidence belts of estimated means (or the regression model) are determined as the first step. The 95% confidence interval can then be used to estimate thresholds, defined as the concentration at which the upper 95% confidence interval of the control responses intersects with the lower 95% intersection of the best-fit regression model.

Numerous advantages are offered by this approach of estimating low-effect concentrations over hypothesis testing to determine a NOEC value. The regression-based approach is a valid procedure for interpolation of effects to untested concentrations, the fit to the data uses the statistical weight of all the data points, and the goodness of fit can be assessed statistically. However, the superiority of the regression-based approach is lost if the concentration-response data obtained from the bioassay are poor [Moore and Caux, 1997]. The authors feel that this may not be such a bad thing:

Using such (poorly defined) data, the conventional hypothesis testing approach would likely determine a NOEC that underestimates chemical toxicity. Thus, the regression-based approach precludes the use of poor quality information, whereas the hypothesis testing approach will not only make use of such information but will do so in the direction that underestimates chemical effects.

4.2.3 Implications for choice of assay - Part two

For the regression-based approach to be employed, there is the need, as before, for the concentration-response relationships of the test agents to be well-characterised, thus ensuring that the fit to the experimental data is statistically valid. In order to ensure this, rather than using the traditional approach of “few doses, many replicates,” the more appropriate protocol would be to include more closely spaced doses and fewer replicates [Moore and Caux, 1997]. As discussed earlier (4.1.4), the large number of data does make a high through-put in vitro bioassay the assay of choice. The reduced variability offered by most in vitro assays compared to that of in vivo assays is also an important consideration.
4.3 Testing mixtures experimentally

It is our intention to characterise concentration-response relationships for numerous xenoestrogens (Chapter 4), and then use that data and the concepts described above to answer the two original questions (Chapters 5 and 6): Are there mixture effects with combinations of xenoestrogens at individually low-effect concentrations? And, what are the consequences of the XEs' joint actions on the potent effects of 17β-estradiol? Clearly, this will involve experimentally investigating various mixtures and comparing the results with our expectation predictions. How?

We described previously (section 4.1), how the application of concentration-response analyses to combinations of two test agents will transform the familiar two-dimensional plots into three-dimensional response surfaces. Conversely, multiple mixtures composed of \( n \) constituents will produce response surfaces in \((n+1)\)-dimensional space. However, representations in two-dimensional space can be achieved in two ways: (i) The concentration of only one component of a multiple mixture is varied while all others are held constant. Or alternatively, (ii) the mixture ratio of a multiple mixture is kept constant, and the effect of varying the total concentration of the mixture is analysed. The second approach lends itself particularly well to our purposes, as it allows the assessment of mixture effects at very low concentrations.

Thus, the task is to record concentration-response curves of individual mixture components, construct multiple mixtures with defined mixture ratios and to establish empirically the concentration-response relationship of the mixtures. Finally, experimental data will be compared with predicted concentration-response curves calculated using the concepts of effect summation, concentration addition and independent action, assuming additivity. This procedure will help to establish whether the combined effects of multiple mixtures can be predicted on the basis of data about the potency of individual mixture constituents, and will give information about the predictive value of the three concepts.

The first issue to deal with before initiating any studies is, of course, the choice of assay system. The remainder of this chapter will investigate its appropriateness for mixture studies, i.e. does it meet the requirements described above, for example, low variability and high through-put?
4.4 Assessing estrogenicity

The meaningful assessment of xenoestrogens is complicated by the fact that synthetic and natural chemicals may theoretically modulate the endocrine system as hormonal mimics, blockers or both [McLachlan, 1993]. Reel et al. [1996], suggest that an assay system should assess whether an estrogen can act through hormone receptors and enhance or diminish the activity normally controlled by the endogenous hormone. As such, an assay system could be useful in determining whether a chemical has hormone modulating activity and at what exposure levels such activities would cause effects in the environment. Others are more specific, favouring in vivo assays as the only valid method to define an endocrine disrupter:

An endocrine disrupting chemical is one that can induce adverse health effects in an intact organism, consequent to disruption of the organism's endocrine system. Other potentially relevant properties of the chemical, including any effects observed in vitro, can only contribute to its definition as a potential endocrine disrupter.

Ashby et al., 1997

In vivo assays are, of course, considered the most relevant and in vivo confirmation of in vitro activity is generally required before some will attribute a given toxic property to the test agent. Their clear advantage over in vitro assays is that they take into consideration the effects of metabolism, plasma-protein binding and pharmacokinetics. Additionally, in combination with a positive control, antiestrogens can also be investigated. However, it should be noted that such assays can be both cumbersome and very costly.

Mammalian estrogenicity assays consider reproductive tract responses and are invariably carried out in rats [Galand, et al., 1987; Branham et al., 1988], or mice [Milligan et al., 1998; Ulrich et al., 2000], that are immature, hypophysectomised or ovariectomised, and test compounds are administered orally, or by subcutaneous or intraperitoneal injection, for hours or days. There are also a variety of end points that are used, depending on whether early or late responses are being studied.

A sensitive in vivo assay using induction of vitellogenin in juvenile rainbow trout has been reported by Sumpter and Jobling [1995]. The relevance of such an assay is clear when one considers that the aquatic environment is a sink for persistent contaminants. As with the mammalian assays, running costs are high, invariably even more so in terms of equipment and the volume of test compound required.
The in vivo assays represent a gold standard in estrogen biological assays, but running costs will, in all but the most special situations, almost certainly prohibit their use for mixture analyses [Thorpe et al., 2001]. In vitro biological assays such as the MCF-7 cell proliferation assay [Soto et al., 1995; Payne et al., 2001], estrogen receptor binding [Ireland et al., 1980], and estrogen receptor-dependent transcriptional expression [Zacharewski et al., 1995; Routledge and Sumpter, 1996], are useful tools for identifying and determining relative potencies of estrogenic chemicals. Not only are these assays relatively inexpensive and simple to run, but they allow large-scale screening in relatively short time periods. An extensive review of the rodent assays as well as the in vitro assays has been carried out by Reel et al. [1996].

As mentioned earlier, our purposes are most likely to be best served by an in vitro assay system that is both high through-put and reproducible. We have chosen to utilise a recombinant yeast estrogen screen (YES), an in vitro screen for agents that are capable of interacting with the α-human estrogen receptor (hERα), which has been shown to meet those requirements [Routledge and Sumpter, 1996].

4.4.1 The yeast estrogen screen (YES)

Receptors linked to reporter genes in transformed cellular systems have been proposed as a useful tool by which chemicals can be defined by their functional properties [McLachlan, 1993]. A number of transformed yeast cell line systems have been developed for the detection of xenoestrogens and other ER studies [McDonnell et al., 1991; Lyttle et al., 1992; Katzenellenbogen et al., 1993; Klein et al., 1994; Routledge and Sumpter, 1996; Arnold et al., 1996b]. The recombinant yeast screen used in these studies was developed in the Genetics Department of what was Glaxo Group Research Ltd., and described in detail by Routledge and Sumpter [1996]. This screen was chosen in preference to the others primarily as a result of its simplicity [Zacharewski, 1997], not requiring a laborious protocol to quantitate the expression of the target gene, e.g. the LacZ gene [Klein et al., 1994; Arnold et al., 1996b].

The “Glaxo” YES utilises yeast cells genetically modified to stably harbour the DNA sequence of hERα. Figure 4.3 provides an overview of the recombinant yeast screen.
Figure 4.1 - Schematic of the YES. The hERα, integrated into the main genome, is expressed (1) in a form capable of binding to estrogen response elements (ERE) within a hybrid promoter on the expression plasmid (2). Activation of the receptor (3), by binding of a ligand causes expression of the reporter gene Lac-Z (4), which produces the enzyme β-galactosidase. This enzyme is secreted into the medium (5), where it acts to convert the chromogenic substrate CPRG (6), into chlorophenol red, a change that is measured by absorbance. Adapted from Routledge and Sumpter [1996].

The YES is ideal for use in the field of combination effects as it is rapid and has been shown to be highly reproducible and sensitive [Beresford et al., 2000; Silva et al., 2001]. As the assay monitors only events immediately following hERα activation, it is impossible to study the effects of converging signalling pathways, feedback loops, etc. The special features of the yeast cell wall and the presence of a selective transporter [Kralli et al., 1995], will almost certainly complicate comparisons with uptake and transport phenomena typical for cell membranes in mammalian cells. In common with many in vitro systems, the assay is unable to model toxicokinetic interactions between test compounds that might occur at higher physiological levels.

4.5 Test agents

The choice of test agents in these studies was made on the basis of our aims; i.e. to investigate the application of models to predict endocrine disrupter mixture effects; and
to determine the plausibility of the claim that low levels of weak xenoestrogens are unlikely to result in detrimental health effects. As such, we chose agents that were known to induce activation of the estrogen receptor, and produced high, near maximal, responses in the YES. The reason for this constraint lies in the mathematical features of the CA concept: CA estimates concentrations of mixtures (and single agents) associated with predetermined effect levels. With estrogenic chemicals that yield only submaximal effects at saturating concentrations, this would lead to mixture effect prediction curves not exceeding the lowest maximal effect of the mixture constituents individually. In many cases this might complicate the assessment of agreement between experimental observation and predicted mixture effects. In order to avoid such ambiguities, only xenoestrogens with maximal effects of at least 60% of those seen with steroidal estrogens were included in mixture studies. Many compounds were taken from the study by Miller and colleagues [2001].

There is one exception, the organochlorine o,p'-DDT, which was chosen deliberately because it failed to meet the criterion of high response. This pesticide has previously been shown, by us [Payne et al., 2000], and others [Coldham et al., 1997], to exhibit a low maximal response in the YES. It was primarily for this reason that the pesticide was selected. It is, however, a very relevant XE in that the ubiquitous organochlorine pesticide is well documented as weakly estrogenic [Soto et al., 1995], and is present in human tissues [Hunter et al., 1997]. The other agents are also relevant, to varying degrees, due to either their commercial use, persistence in the environment or presence in diet. However, it should be stated that the level of exposure to many of these agents is not yet known.

### 4.6 Experimental procedure

#### 4.6.1 Chemicals

17β-Estradiol (98% pure), and genistein (98% pure) were purchased from Sigma Chemical Company Ltd. (Dorset, UK), resorcinol monobenzoate (99% pure), phenyl salicylate (99% pure), benzyl-4-hydroxyparabene (99% pure) and 2,4-dihydroxybenzophenone (99% pure) from Aldrich Chemicals (Dorset, UK), bisphenol A (4,4'-isopropylidene-diphenol; 97% pure) from Acros Organics (Geel, Belgium), 4'-
chloro-4-biphenylol (>95% pure), 2,3,4-trichlorobiphenyl (99.9% pure), 2',5'-dichloro-4-biphenylol (>95% pure), 2,3,4,5-tetrachlorobiphenyl (>97% pure) and 2',3',4',5'-tetrachloro-4-biphenylol (95% pure) from Ultra Scientific (North Kingston, RI, USA).

The agents were used as supplied and 1 mM stock solutions prepared in HPLC-analysed absolute ethanol (Mallinckrodt Baker, Deventer, Holland). Stocks and subsequent dilutions were kept in critically cleaned glass containers and stored at -20°C. All other chemicals used were research grade from Sigma Chemical Company Ltd. (Dorset, UK), unless otherwise stated.

4.6.2 The recombinant yeast estrogen screen

A detailed description of the yeast estrogen screen can be found in Routledge and Sumpter [1996]. Critical steps were carried out in a laminar air flow cabinet. Briefly, 50 mL of growth medium were inoculated with 125 μL of 10× concentrated yeast stock and grown overnight in an orbital shaker at 28°C until turbid (absorbance at 640 nm of 1.0). The assay medium consisted of 50 mL of growth medium, chlorophenol red-β-galactopyranoside (10 mg/L, CPRG, Boehringer Mannheim, East Sussex, UK) and 2 mL of the overnight yeast culture.

Single agents stock solutions were serially diluted in HPLC-analysed ethanol. Aliquots of 10 μL of the dilutions were transferred to 96-well optically flat bottom microtiter plates and allowed to evaporate to dryness. All plates included a row of ethanol controls (i.e. no test agent) and a row of assay medium without yeast cells (blanks). To each well, except the blanks, a volume of 200 μL of yeast seeded assay medium was added. To minimise evaporation during the subsequent incubation time, the outer wells were not used for test agents, instead being filled with sterile UHQ water. Plates were shaken vigorously for 2 minutes on a microtiter plate shaker, before incubating at 32°C in a humidified box for 72 h. During this period they were again shaken at 24 h and 71 h. Plates were then analysed spectrophotometrically at 540 nm (colour) and 620 nm (turbidity) using a Labsystem Multiskan Multisoft plate reader. Data shown in graphs are corrected for turbidity and constitutive Lac-Z expression seen in the ethanol treated controls as follows:

\[
\text{Corrected absorbance} = \text{test } 540 \text{ nm} - \text{test } 620 \text{ nm} + \text{control } 620 \text{ nm} - \text{control } 540 \text{ nm}
\]
Single agent data were obtained from at least two experiments run in duplicate. Data from replicate experiments were pooled if the 95% confidence intervals for each dose tested overlapped. Nominal concentrations were used.

4.6.3 Dosimetry

Scatter plots of corrected absorbance values ("effect") versus log concentration were constructed and analysed using the best-fit approach [Grimme et al., 1998; Scholze et al., 2001]. The best-fit from a number of non-linear regression models was selected, using the goodness of fit criterion of sum of absolute errors being greater than 0.95, for final data analysis. In these studies the model offering best description of the concentration-response relationships was the asymmetric (or three parameter) Hill function;

\[
\text{Effect} = \text{Min} + \frac{\text{Max} - \text{Min}}{1 + \left(\frac{c}{\text{EC}_{50}}\right)^{-p}}
\]

where Min and Max are the minimal and maximal observed effects, respectively, c the concentration of test agent, EC\(_{50}\) the concentration of test agent yielding half maximal effects and p the slope parameter.

The 95% confidence intervals of the best estimate of mean effects were also calculated. Non-linear curve-fitting was carried out using SigmaPlot (v. 5.0, SPSS Inc. US).

4.6.4 NOECs, EC\(_x\) point estimates and thresholds

No Observed Effect Concentrations (NOECs), the highest tested concentration at and below which the effect parameter does not differ statistically from the effect parameter observed in the control, were established for each test agent.

A simple analysis of variance (ANOVA) was used to compare all of the treatment groups, including the control group. ANOVA is permitted when the variances are homogeneous, which was confirmed using Bartlett’s test for homogeneity of variances [Sokal and Rohlf, 1981], and the data are independent and normally distributed.
(Kolmogorov-Smirnov test). A finding, with ANOVA, of "significant difference among the groups being compared" indicated the need for further (post hoc) testing to establish which groups differ significantly from which other groups. A number of post hoc tests are available and of those the most commonly used in toxicology are, Duncan's multiple range test, Scheffe's test, Dunnett's t-test, and Williams' t-test. The most appropriate of those tests for our purposes is the Dunnett's t-test [Dunnett, 1955; 1964], which allows multiple comparisons of treatments versus control and does not require that the control group be of the same sample size as the treated groups.

Briefly, in a study with $K$ groups (including the control) we will make $(K-1)$ comparisons. The Dunnett's $t$-distribution is predicated by the assumption that a test level is set for the entire set of $(K-1)$ decisions and not for each comparison. To use the Dunnett's $t$-table [Bechhofer and Dunnett, 1988], the parameters required are $K$ and the number of degrees of freedom for the mean square within group ($MS_{wg}$). The test value is given by,

$$ t = \frac{|T_j - T_i|}{\sqrt{2MS_{wg} / n}} $$

where $n$ is the number of observations in each of the groups, $T_j$ is the mean of the control group, and $T_i$ is the mean of the appropriate treatment group.

As well as the NOEC value for each test agent, we determined the various EC$_x$ point estimates, i.e. EC$_{01}$ and EC$_{50}$ values. This involves interpolating or extrapolating the concentration of test agent that results in 1% or 50% of the maximal response in the YES, i.e. an effect equivalent to 1% or 50% of 1.7 a.u., from the biometrical concentration-response analysis. Thresholds were also estimated using the regression-based approach (as defined in 4.2.2).

4.7 Results

4.7.1 Overview

All tested agents induced activation of the hER$\alpha$ in a concentration-dependent manner. Figures 4.4 to 4.12 show the scatter plots and best-fit regression models for 17$\beta$-estradiol and twelve xenoestrogens (including the phytoestrogen genistein). The
reproducibility achievable in the YES was high. The best-fit regression lines of all but one (o,p'-DDT), of the tested chemicals were of relatively similar shapes and slopes. There were considerable variations in potency. For example, 17β-estradiol was approximately 340-times more potent than 2',3',4',5'-tetrachloro-4-biphenylol and over 300000-times more potent than phenyl salicylate, the weakest of all tested agents. The concentration-response data for 2,3,4-trichlorobiphenyl and phenyl salicylate do not show the levelling-off normally expected to occur at higher concentrations as, due to concerns of limited aqueous solubility, we did not test concentrations above 100 μM.

4.7.2 17β-estradiol

The scattergram routinely obtained for the endogenous hormone 17β-estradiol is shown in Figure 4.4. The experimental data, in this instance, were fitted to the asymmetric Hill function. This provided a best-fit regression line with a maximal absorbance of 1.657 units (corrected for readings in untreated controls), and a median effect concentration (EC₅₀) of 180 pM. The parameters of the best-fit regression models along with the NOECs, ECₙ₀ values and thresholds are summarised at the end of the results section for 17β-estradiol and the other test agents in Table 4.1.

![Figure 4.4 - Concentration-response relationship for 17β-estradiol in the YES.](image)
4.7.3 Phenolic additives

A common feature of many compounds that bind with the estrogen receptor is a phenolic group [Katzenellenbogen, 1995]. Previous studies carried out by other groups investigated a number of phenolic additives for estrogenicity. A large number were positive and some were relatively potent compared to other xenoestrogens [Schultz et al., 2000; Miller et al., 2001]. As a result they were a natural choice for our studies.

Bisphenol A (BPA), is a monomer in polycarbonate plastics and a constituent of epoxy and polystyrene resins found extensively in food packaging and dental sealants. Human exposure to BPA is significant [Brotons et al., 1995; Olea et al., 1996], and its estrogenic activity is well established [Dodds and Lawson, 1936; Steinmetz et al., 1997]. In the YES the response to BPA (Figure 4.5) was in agreement with those previously reported [Routledge and Sumpter, 1996; Gaido et al., 1997].

![Figure 4.5 - Concentration-response analysis for bisphenol A.](image)

Experiments were carried out in duplicate and repeated 3 times. Data were fitted to the best-fit regression model (asymmetric Hill function). For explanation of symbols, see legend to Figure 4.4.

Four other phenolic additives from two distinct classes were investigated. 2,4-Dihydroxybenzophenone, resorcinol monobenzoate, and phenyl salicylate (Figure 4.6), are UV-screening agents used in the cosmetics and polymers industries whilst benzyl-4-hydroxyparabene (Figure 4.7) is used as a preservative [Miller et al., 2001].
Figure 4.6 - Dose-response analyses of three UV-screening agents in the YES. Data for 2,4-dihydroxybenzophenone (top), resorcinol monobenzoate (middle), and phenyl salicylate (bottom), are fitted to the asymmetric Hill function. Experiments run twice at least in duplicate. For explanation see legend to Figure 4.4.

As mentioned earlier, phenyl salicylate failed to exhibit the complete levelling-off normally expected to occur at higher concentrations as, due to concerns of limited aqueous solubility, we did not test concentrations above 100 μM. The concentration-response analyses obtained for all tested phenolic additives was in broad agreement with previously published data [Miller et al., 2001]. In terms of potency relative to 17β-
estradiol, the most potent were 2,4-dihydroxybenzophenone and benzyl-4-hydroxyparabene at 3900- and 4400-times less potent than the hormone, respectively. Phenyl salicylate, on the other hand was approximately 300000-times less potent than 17β-estradiol.

![Graph](image)

**Figure 4.7 - Dose-response relationship for benzyl-4-hydroxyparabene in the YES.** Data are from three separate experiments run in duplicate and were fitted to the asymmetric Hill dosimetric function. See Figure 4.4 legend for explanation.

The slopes of the best-fit regression models to the data obtained from the phenolic additives were relatively congruent, (the slope given by p in the asymmetric Hill function, ranging from 2.355 to 2.983). The important parameters of the regression models are summarised in Table 4.1.

### 4.7.4 Genistein

Genistein (4’,5,7-trihydroxyisoflavone), a phytoestrogen, was observed to be estrogenic in the 1950s when it was noticed that the reduced reproductive capacity in sheep was due to their grazing on genistein-rich clover.

A great deal of data exist on genistein’s apparent duality. The data indicate that genistein can act as both an estrogen and an anti-proliferative agent [Reviewed extensively by Bouker and Hilakivi-Clarke, 2000].

Withstanding the controversy surrounding genistein, it is clear that it acts via the estrogen receptor, as demonstrated in Figure 4.8 and by the ability of the antiestrogen
ICI 182,870 to inhibit increases in uterine vascular permeability [Milligan et al., 1998]. Asymmetric Hill function parameters for the best-fit regression model to the data (Table 4.1) show that genistein is approximately 3000-fold less potent that 17β-estradiol, slightly less potent than reported by Coldham et al. [1997], using the yeast estrogen screen described by Klein et al. [1994].

![Figure 4.8 - Concentration-response analysis for the phytoestrogen genistein. Data, from three experiments run in duplicate, (blue circles) are fitted to the asymmetric Hill function (black sigmoidal line). The grey band is the 95% confidence belt of mean responses.](image)

4.7.5 Polychlorinated biphenyls (PCBs) and hydroxylated PCBs

Prior to the ban on their production, polychlorinated biphenyls (PCBs) were widely used in a variety of industrial and consumer products. These halogenated aromatic compounds are frequently found as complex mixtures of congeners, comprising theoretically 209 congeners [Brouwer et al., 1999]. PCBs are ubiquitous, highly persistent environmental contaminants identified in almost every component of the global ecosystem [WHO, 1993]. Their lipophilic character permits concentration in the food chain and exposure has been seen in terrestrial and marine mammals [Kannan et al., 1989].

PCBs have been implicated in a broad range of biochemical and toxic effects [Mcfarland and Clarke, 1989]. In the late 1980s attention turned to consider the estrogenicity of PCBs and their hydroxylated metabolites [Korach et al., 1988; Garner et al., 1999]. Based on reports in the literature, we decided to test two PCBs and three hydroxy-PCBs (Figure 4.9) in the YES [Coldham et al., 1997].
Figure 4.9 - Polychlorinated biphenyls (PCBs) and hydroxy-PCBs investigated using the YES.

Figure 4.10 shows the concentration-response analyses for 2,3,4-trichlorobiphenyl and 2,3,4,5-tetrachlorobiphenyl. The dose-response analyses of the three hydroxy-PCBs are presented in Figure 4.11.
The phenolic metabolites of PCBs were more potent than the tested PCBs, 2,3,4-trichlorobiphenyl and 2,3,4,5-tetrachlorobiphenyl. The least potent hydroxy-PCB (4'-chloro-4-biphenylol) was approximately 27500-fold less potent than 17\beta-estradiol, whereas, the most potent of the two tested PCBs (2,3,4,5-tetrachlorobiphenyl) was 3300-fold less potent than the hormone (Table 4.1).

![Figure 4.11](image-url)

**Figure 4.11 - Response of the YES to three hydroxylated polychlorinated biphenyls.** Top: 4'-chloro-4-biphenylol, middle: 2',5'-dichloro-4-biphenylol, and bottom: 2',3',4',5'-tetrachloro-4-biphenylol. Data (from at least two experiments run in duplicate), are fitted to the asymmetric Hill function in all three cases. See Figure 4.4 for details.
4.7.6 2,2-Bis(p-chlorophenyl)-1,1,1-trichloroethane (DDT)

In 1972 the United States banned the production of DDT, and in the 1980s Third World countries began to ban its use in agriculture. However, its use in vector control, (i.e. against malaria-bearing mosquitoes) is still widespread in, for example, India [IARC, 1991]. As discussed in section 1.2.2, reproductive malformations in birds, including the feminization of male Channel Island gulls of the south coast of California, have been attributed to contamination by environmentally persistent DDT [Fry and Toone, 1981; Fry, 1995].

Due to its nature, DDE, the prevalent metabolite of DDT, persists in the environment and is concentrated in the food chain [Travis and Arms, 1988], predominantly in adipose tissue [Waliszewski et al., 1996]. Therefore, it is detected in human breast milk [Hooper et al., 1998; Lutter et al., 1998], and cow’s milk [Waliszewski et al., 1996b].

Some congeners of DDT (o,p’-DDT and p,p’-DDT), are able to induce cell proliferation in estrogen-dependent breast cancer cells [Soto et al., 1995], as is p,p’-DDE [Bustos et al., 1988]. Shekhar et al. [1997], showed o,p’-DDT to be more efficient in inhibiting E2 binding to the ER than p,p’-DDT, and our laboratory has recently shown o,p’-DDT to be the most potent DDT congener using the endpoint of MCF-7 breast cancer cell proliferation [Payne et al., 2001].

In the YES, o,p’-DDT produced a shallower concentration-response curve than the other tested compounds (Figure 4.12), as we have previously published [Payne et al., 2000], however, this response occurred at lower concentrations than reported by

![Figure 4.12 - Concentration-reponsne analysis for o,p'-DDT in the YES. Data, fitted to the asymmetric Hill function, are from two experiments run in duplicate. See Figure 4.4 for details.](image)
Routledge and Sumpter [1996]. The median effect concentration for o,p'-DDT was 2.2 μM and the maximal effect produced was 0.45 a.u. (Table 4.1).

4.7.7 Summary

The characteristics of the concentration-response analyses for the twelve xenoestrogens and 17β-estradiol are summarised below (Table 4.1). The table also shows the important low-effect concentrations, i.e. NOEC, EC01, and threshold values.

Table 4.1 - Summary of parameters for test agents in the YES

<table>
<thead>
<tr>
<th>Compound</th>
<th>NOEC (μM)</th>
<th>EC01 (μM)</th>
<th>Threshold (μM)</th>
<th>X50 (μM)</th>
<th>p</th>
<th>Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>17β-estradiol</td>
<td>1.15x10⁻⁵</td>
<td>2.3x10⁻⁵</td>
<td>3.1x10⁻⁵</td>
<td>1.8x10⁻⁴</td>
<td>2.273</td>
<td>1.657</td>
</tr>
<tr>
<td>bisphenol A</td>
<td>0.4215</td>
<td>0.632</td>
<td>0.692</td>
<td>2.879</td>
<td>2.983</td>
<td>1.676</td>
</tr>
<tr>
<td>benzyl-4-hydroxyphenol</td>
<td>0.1164</td>
<td>0.130</td>
<td>0.148</td>
<td>0.787</td>
<td>2.519</td>
<td>1.703</td>
</tr>
<tr>
<td>2,4-dihydroxybenzophenone</td>
<td>0.0728</td>
<td>0.124</td>
<td>0.139</td>
<td>0.702</td>
<td>2.617</td>
<td>1.713</td>
</tr>
<tr>
<td>resorcinol monobenzoate</td>
<td>0.9898</td>
<td>2.236</td>
<td>2.442</td>
<td>13.49</td>
<td>2.546</td>
<td>1.765</td>
</tr>
<tr>
<td>genistein</td>
<td>0.038</td>
<td>0.086</td>
<td>0.095</td>
<td>0.595</td>
<td>2.337</td>
<td>1.672</td>
</tr>
<tr>
<td>2,3,4-trichlorobiphenyl</td>
<td>1.501</td>
<td>2.420</td>
<td>2.797</td>
<td>30.13</td>
<td>1.755</td>
<td>1.522</td>
</tr>
<tr>
<td>2,3,4,5-tetrachlorobiphenyl</td>
<td>0.2945</td>
<td>0.177</td>
<td>0.217</td>
<td>4.921</td>
<td>1.324</td>
<td>1.493</td>
</tr>
<tr>
<td>4'-chloro-4-biphenolol</td>
<td>0.054</td>
<td>0.074</td>
<td>0.086</td>
<td>0.599</td>
<td>2.161</td>
<td>1.665</td>
</tr>
<tr>
<td>2',5'-dichloro-4-biphenolol</td>
<td>0.011</td>
<td>0.011</td>
<td>0.012</td>
<td>0.060</td>
<td>2.699</td>
<td>1.713</td>
</tr>
<tr>
<td>2',3',4',5'-tetrachloro-4-biphenolol</td>
<td>0.005</td>
<td>0.008</td>
<td>0.009</td>
<td>0.061</td>
<td>2.202</td>
<td>1.711</td>
</tr>
<tr>
<td>o,p'-DDT</td>
<td>0.585</td>
<td>0.572</td>
<td>0.761</td>
<td>2.157</td>
<td>2.429</td>
<td>0.425</td>
</tr>
</tbody>
</table>

* Defined in Experimental procedure. Arbitrary units for Max
* No Observed Effect Concentration, as described in 4.6.4.
* Concentration producing effect 0.017 a.u., i.e. EC01 of maximal response in YES.
* Defined as the intersection of the upper 95% confidence limit (CL) of the mean response of controls (0 ± 0.018, n = 20) with the lower 95% CL of the best fit regression model.
* Data obtained using earlier batch of long term yeast stock.

It is immediately apparent that, with the exception of o,p'-DDT, the maximal responses of the test agents were very similar, with phenyl salicylate giving the lowest maxima, some 70% of the maximum response of resorcinol monobenzoate. Also, very striking is the difference in potency of 17β-estradiol compared with all of the XEs. The
EC₅₀ of the most potent XE (2',5'-dichloro-4-biphenylol), is approximately 350-fold lower than the equivalent benchmark for 17ß-estradiol.

Encouragingly, both the NOEC and EC₀₁ values were below the statistical thresholds, except in the case of 2,3,4,5-tetrachlorobiphenyl’s NOEC value which was higher. In the light of preceding descriptions of estimates of low-effect concentrations, the fact that the EC₀₁ values were above the NOEC values for all but three XEs was an interesting outcome to be discussed in more detail below.

4.7.8 Reproducibility

The variability of the data obtained from a single experiment was minimal. The inter-experimental variability was also very small. The data obtained over two separate occasions for 2',3',4',5'-tetrachloro-4-biphenylol is typical of the reproducibility of the yeast estrogen screen (Figure 4.12). Results obtained from different long-term stocks were found to exhibit different responses (data not shown), and so data were not pooled as this impacted negatively on the regression fits. Instead, for a given mixture study, all of the single agents and the mixture would be tested using the same batch of long-term yeast stock.

![Figure 4.12 - Inter-experimental variability of data obtained in the YES for 2',3',4',5'-tetrachloro-4-biphenylol. Replicate data (green and blue circles) for experiments run on separate occasions. The regressions fit to the separate experiments using the asymmetric Hill function are also shown.](Image)
4.8 Discussion

In order to achieve our ultimate aim of investigating the joint action of low levels of xenoestrogens and determining the impact of these weak XEs on the effect of 17β-estradiol, the assay system chosen needed to fulfil certain requirements. The criteria that have to be satisfied have been described in some detail earlier (section 4.1). Firstly, the logistics of carrying out a systematic analysis of mixtures of ten or more components means that vast amounts of data will be produced. For reasons of cost and ethics a high through-put in vitro estrogenicity assay was the only viable choice. Secondly, as the focus would be on low-effect concentrations, the assay must be reproducible and sensitive. In this chapter we have investigated whether the recombinant yeast estrogen screen [Routledge and Sumpter, 1996], meets these criteria.

From the literature, it was clear that the YES met the first requirements. The assay is carried out in 96-well microtitre plates, allowing five test agents to be tested at ten concentrations on one plate, and results are obtained in five days. The ability to test a compound at ten finely spaced concentrations ensures the dose-response relationship is well characterised, especially at the points of inflection. In terms of the reproducibility of the YES, the data obtained with 17β-estradiol and twelve xenoestrogens exhibited low inter-experimental variability. In the worst case, o,p'-DDT, the variation between replicate experiments approached 15%. The impact of this low variability and ten concentrations being tested was demonstrable by the goodness of fit for the regression models, defined by the sum of absolute errors, being greater than 0.95 in all cases.

The significance of the highly reproducible data obtained with the YES was evident in other aspects too. Moore and Caux [1997], found that the analysis of poor quality data to determine No Observed Effect Concentrations would lead to gross underestimation of chemical effects. Using the Dunnett's t-test we established NOEC values for all of the tested compounds and compared these to the EC01 values, which are a suggested statistical operationalisation of NOECs. Due to the numerous recent criticisms of NOECs [Chapman et al., 1996; Moore and Caux, 1997], we had the preconceived belief that the NOECs would be markedly higher than the EC01 values and would also exceed threshold values. In fact, this was not the case. The robustness of the experimental data meant that the NOECs were below EC01s in the majority of cases.
And in the one case where the NOEC exceeded the threshold 2,3,4,5-
tetrachlorobiphenyl, an explanation is forthcoming. In this instance, although the
goodness of fit is extremely high (>0.99), the low concentrations appear only
reasonably modelled by the regression (Figure 4.10), with the regression lying at higher
effects than the observed data. Thus, the EC_{50} and threshold lie to the left, i.e. lower
concentrations, than the NOEC. In the light of this one apparent failure it should not be
overlooked that, in the main, if the data are from a reproducible assay and the
concentrations tested are well considered, NOEC values may still be valid.

Turning our attention to the estrogenicity of the test agents, the greater potency
of 17β-estradiol compared to the XEs is clear. The twelve XEs ranged from 330-fold
less potent (2',5'-dichloro-4-biphenylol), to approximately 300 000-fold less potent
(phenyl salicylate) than the hormone. Based on these results, it is understandable why
many researchers are sceptical about the impact these agents can have on an organism
when the endogenous steroidal estrogens are so much more potent. In the next chapter
we will investigate whether those sceptics are correct in believing that XEs are too weak
to modulate the effects of 17β-estradiol.
CHAPTER 5 - DEFINING THE IMPACT OF WEAKLY ESTROGENIC CHEMICALS ON THE ACTION OF STEROIDAL ESTROGENS

An impasse appears to have been reached in the field of xenoestrogens. We earlier described how, although increasing numbers of environmentally relevant compounds are being identified as estrogenic, their low potency, together with the low levels at which they are found in human tissues, are believed to indicate “no risk.” Their low potency in relation to 17β-estradiol is often used to argue that XEs in combination with steroidal estrogens will not produce effects distinguishable from those of the steroid [Safe, 1995b]. There is, however, little empirical evidence to substantiate this belief.

To help solve this dilemma, we set out to evaluate whether synthetic estrogenic chemicals, when combined with 17β-estradiol, would contribute to the overall mixture effect. Our main interest was to define factors that may influence the ability of a weakly estrogenic compound to modulate the effects of the hormone 17β-estradiol. We hypothesised that the potential impact of a weak xenoestrogen on 17β-estradiol would predominantly depend on (i) its concentration relative to the steroid hormone, i.e. the mixture ratio and (ii) its relative potency.

A crucial part of studying combination effects is the definition of what response is expected from a particular mixture. In Chapter 4 we discussed three widely used concepts that utilise individual component’s dose-response data and the composition of the mixture to predict the expected (additive) mixture effect. The application of these concepts, effect summation, concentration addition and independent action, will often provide differing additivity references [Greco et al., 1992], and therefore it is important to consider which model is most appropriate. Previously, we tested numerous xenoestrogens individually in the YES and demonstrated that the dose-response relationships were all sigmoidal. This fact has immediate consequences on the use of one particular concept. Effect summation (ES), only gives meaningful additive predictions when applied to mixtures of agents that exhibit linear concentration-response relationships (see 4.1.7). Clearly, the only expectation, on the researchers part, from the use of ES in assessing XE mixtures should be that it will provide a totally unreliable prediction! In terms of concentration addition (CA) and independent action...
disputes still exist as to their general applicability [Greco et al., 1992], and yet there is no criterion that would help decide \textit{a priori} which of the two concepts should be applied. In the following studies, therefore, both concepts are used side-by-side.

To test our hypotheses that the mixture ratio and relative potency of the xenoestrogens compared to 17\beta-estradiol will significantly influence the joint action of a mixture, bisphenol A (BPA) and \textit{o,p'-DDT}, in combination with 17\beta-estradiol, were selected for in-depth studies. We previously observed that \textit{o,p'-DDT} produced a dose-response curve dissimilar to those of the other agents, typified by 17\beta-estradiol and BPA. The organochlorine reached a maximal response less than 50\% of the maximal response of 17\beta-estradiol. The inclusion of \textit{o,p'-DDT} in these studies offered the opportunity to investigate how the modelling concepts differed when faced with a mixture component with sub-maximal effects.

The fixed mixture ratio design described by Altenburger et al. [2000], and adopted in our earlier work on xenoestrogen mixtures [Payne et al., 2000], lends itself particularly well to achieve the goals of the present studies (see section 4.3). The fact that this experimental design is particularly useful for investigating low effect concentrations represents a major improvement over the "classical" pharmacological design described by Pöch [1993]. Using the "classical" approach, the concentration of one agent is held constant whilst the effect of a second agent is studied by varying its concentration. Thus, the study of low concentrations of all mixture components is not possible because one agent is always present at a concentration that produces significant effects.

\textbf{5.1 Experimental procedure}

The yeast estrogen screen (YES) was carried out exactly as described previously in \textbf{Chapter 4}. Stock solutions of the binary mixtures were made up at 1 mM, and stored as the single agents, at -20\textdegree C.

In terms of dosimetry, analysis of the scattergrams was also carried out as described earlier. In the studies presented in this chapter, we found that the non-linear regression models that provided best fits to our data, based on the sum of absolute errors, were the asymmetric Hill function and the three-parameter Gompertz model. The
asymmetric Hill function was detailed in section 4.6.3. The formula for the three-parameter Gompertz function is

\[
\text{Effect} = \text{Max} \times e^{-e^{(c-\text{EC}_{50}/b)}}
\]

where Max denotes the maximal observed effect, \(c\) is the concentration of test agent, \(\text{EC}_{50}\) is the concentration of test agent yielding half maximal effects and \(b\) is the slope parameter.

5.1.1 *Calculation of predicted mixture effects*

Once concentration-response curves for \(E_2\), \(o,p'\)-DDT and BPA had been established, the responses of binary mixtures of one xenoestrogen with \(E_2\) were predicted assuming additive combination effects. The expected effects for a range of mixture ratios were calculated using the models described below.

The model of concentration addition (CA) estimates concentrations of agents that yield a predetermined effect. Such estimates are derived from the relative prevalence of each agent in the mixture and from data on the concentrations of the mixture components that individually would produce this same predetermined effect. Thus, assuming that the combined effect of the mixture is additive, the following expression will hold for any effect level:

\[
\sum \frac{c_i}{\text{EC}_i} = 1 \quad (5.1)
\]

where \(c_i\) denotes the concentration of the agent \(i\) in a mixture yielding an effect \(E\) and \(\text{EC}_i\) the concentration of \(i\) needed to produce effect \(E\) on its own. With synergistic mixture effects the expression will give values <1, with antagonistic mixture effects >1 [Berenbaum, 1985].

Eq. (5.1) implicitly defines effect concentrations of a mixture of \(n\) agents. It can be used to calculate mixture concentrations that produce a predetermined effect provided the \(\text{EC}_i\) of the individual mixture components and their prevalence in the
mixture are known. Thus, the concentration $c_i$ of agent $i$ in the mixture is related to the
total mixture concentration by;

$$c_i = p_i \times E_{C_{mix}}$$

where $p_i$ is the concentration of $i$ relative to the total mixture concentration and $E_{C_{mix}}$
the total concentration of the mixture required to produce effect $E$. Substitution of $c_i$ in
Eq. (5.1) gives

$$\sum \left( p_i \times E_{C_{mix}} \right) \over E_{C_i} = 1$$

and rearranging yields

$$E_{C_{mix}} = \left[ \sum \left( p_i \over E_{C_i} \right) \right]^{-1}.$$

The parameters $E_{C_i}$ were calculated from the concentration-response curves of
single agents by using the inverse expression of the asymmetric Hill function or three-
parameter Gompertz model, as appropriate.

The model of independent action (IA) allows predicted effects of mixtures of
known composition to be calculated using the expression;

$$1 - \prod \left[ 1 - E(c_i) \right], \quad (5.2)$$

where $E(c_i)$ is the effect $E$ produced by compound $i$ at concentration $c$. Inherent in this
expression is the fact that $E(c_i)$ cannot exceed 1, i.e. $E(c_i)$ is a fraction of a maximal
possible effect, making independent action a probabilistic model.

Thus, when applying this model to our assay effects $AE(c_i)$, a maximal effect
$E_{max}$ has to be defined. The maximal absorbance value obtained in these investigations
was to saturating concentrations of BPA, and this was chosen as a reference point and
the effects of test agents are expressed relative to the maximal effect of BPA:

$$E(c_i) = {AE(c_i) \over E_{max}}$$
If the concentration-response relationships of all mixture constituents i are described by an appropriate regression model $F_i$ (Hill or Gompertz), the assay effect $AE(c_i)$ can be estimated from the mean effect $F_i(c_i)$ predicted by the regression model. Thus,

$$AE(c_i) = F_i(c_i), \quad \text{and} \quad E(c_i) = \frac{F_i(c_i)}{E_{\text{max}}}$$

and substitution of $E(c_i)$ in Eq. (5.2) yields

$$e_{\text{mix}} = 1 - \prod \left[ 1 - \frac{F_i(c_i)}{E_{\text{max}}} \right], \quad (5.3)$$

In order to ensure comparability of the independent action predictions with those of concentration addition the fractional effects in Eq. (5.3) were rescaled by multiplication with $E_{\text{max}}$, thus:

$$E_{\text{mix}} = E_{\text{max}} \times e_{\text{mix}}, \quad \text{and} \quad E_{\text{mix}} = E_{\text{max}} \left[ 1 - \prod \left[ 1 - \frac{F_i(c_i)}{E_{\text{max}}} \right] \right]$$

### 5.1.2 Assessing mixture predictions

To test the validity of the modelling, two $E_2$-BPA and three $E_2$-o,p'-DDT mixtures with differing mixture ratios were investigated experimentally. Master stocks (1 mM) were made and serially diluted to cover the range of concentrations modelled in the predictions.

As in the case of the single agent data, mixture observations were fitted to various non-linear regression functions and the model yielding the best fit, determined by goodness of fit, was used. The 95% confidence belts of estimated means were also calculated and are presented in subsequent graphs as grey bands around the best fit to the data. The criterion used for deciding agreement between observations and prediction was overlap between the prediction curves and the 95% confidence belts of the best fit regression functions.
5.2 Results

5.2.1 Concentration-response analysis for the single agents

As shown in Chapter 4, each of the single agents, 17β-estradiol, bisphenol A and $o,p'$-DDT induced activation of the hERα in a concentration-dependent manner. The single agent data used in these mixture studies are shown in Figure 5.1. The data are from different experiments to those presented in the previous chapter. Experimental data were fitted to dosimetric functions (three-parameter Gompertz for the E$_2$ and BPA studies; asymmetric Hill function for the E$_2$ and $o,p'$-DDT experiments).

![Figure 5.1](image)

The data presented here were obtained using two different long-term stocks of yeast, i.e. BPA and 17β-estradiol and subsequent mixtures of the two studied with one batch, and the $o,p'$-DDT and 17β-estradiol observations and those of their mixtures were from another. This demonstrates the point made in 4.7.7 that reproducibility between batches was sometimes variable and that, therefore, mixture studies should be undertaken using only one long-term stock of yeast to investigate the single agents and the mixtures. By doing this, the inherent reproducibility of the YES using one batch of long term stock, will ensure that valid conclusions are made from the comparison of mixture predictions with observations and that any observed differences are not due to variations in the assay system. A summary of the single agent concentration-response
relationship parameters, as given by their respective best fit regression models, is given in Table 5.1.

Table 5.1 - Parameters of best fit regression models to observed data for 17β-estradiol, bisphenol A and o,p'-DDT in the yeast estrogen screen.

<table>
<thead>
<tr>
<th>Compound</th>
<th>E2</th>
<th>BPA</th>
<th>E2</th>
<th>o,p'-DDT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batch a 3a</td>
<td>3a</td>
<td>3a</td>
<td>2d</td>
<td>2d</td>
</tr>
<tr>
<td>Regression b</td>
<td>Gompertz</td>
<td>Gompertz</td>
<td>Hill</td>
<td>Hill</td>
</tr>
<tr>
<td>EC50 (μM)</td>
<td>1.59x10⁻⁴</td>
<td>3.9265</td>
<td>2.1x10⁻⁴</td>
<td>2.157</td>
</tr>
<tr>
<td>Slope parameter c</td>
<td>1.29x10⁻⁴</td>
<td>2.173</td>
<td>2.028</td>
<td>2.429</td>
</tr>
<tr>
<td>Max</td>
<td>1.586</td>
<td>1.647</td>
<td>1.148</td>
<td>0.4245</td>
</tr>
</tbody>
</table>

a Batch of long term yeast stock
b Non-linear regression model with best fit to the data
c b for the Gompertz function (5.1) and p for the asymmetric Hill function (4.6.3)

5.2.2 Predicting the effects of binary mixtures at different mixture ratios

The parameters of the best-fit regression lines of the single agents (Table 5.1) were used to calculate predictions for a number of mixtures with varying mixture ratios, assuming additive combination effects (Figure 5.2 and 5.3). Predicted mixture responses were plotted against the sum of the concentrations of both mixture components (curves on the right of each panel). For both the 17β-estradiol-BPA and 17β-estradiol-o,p'-DDT mixtures the figures show shifts of the predicted mixture concentration-response curves towards lower concentrations, which became more pronounced with increases in the relative proportions of 17β-estradiol in the mixtures. Thus, the models predict that the presence of 17β-estradiol should increase the potency of the mixtures, to the point where the hormone swamps completely the effects of BPA or o,p'-DDT.

To highlight the contributions of the less potent xenoestrogens to the overall mixture effect, the predicted responses were also plotted against the 17β-estradiol content of the mixtures (curves on the left of each panel) and compared to the effects caused by the hormone on its own. With mixtures containing larger amounts of the hormone the predicted differences between the effects of the mixture and those of 17β-estradiol were small. For example, the responses calculated for the 1:5000 (17β-estradiol:BPA, 17β-estradiol:o,p'-DDT) mixtures (molar ratios) were almost
indistinguishable from those of 17β-estradiol, indicating that the impact of the XE was negligible. However, the combination effects increased markedly as the composition of the mixture changed in favour of the weaker xenoestrogens. At mixture ratios of 1:20000 (17β-estradiol:BPA, 17β-estradiol:o,p'-DDT) and higher the predicted combination effects were considerably larger than the response expected on the basis of the 17β-estradiol content of the mixture. Here, the weak xenoestrogens are expected to contribute significantly to the overall mixture effect.

Although the two models yielded very similar curves for the 17β-estradiol-BPA mixtures, the CA model consistently produced the more conservative predictions, independent of mixture ratio (Figure 5.2). On the basis of the model predictions we chose to test experimentally the 1:20000 and 1:50000 (17β-estradiol:BPA) mixtures.

Figure 5.2 - Predictions as given by concentration addition (top panel) and independent action (bottom panel) for various molar mixture ratios of E2-BPA. Curves on the right of each panel are predictions against the sum of the concentrations of both mixture components. To highlight the contribution of the weak XE, mixture effects have also been plotted against E2 concentration in the mixture (curves on left of each panel. Mixture ratios are 1:5000 (pink), 1:20000 (green), 1:50000 (blue), and 1:100000 (orange).
The predictions for the mixtures of 17β-estradiol and \( o,p' \)-DDT demonstrated clearly the main difference in the two models, namely that the CA model is unable to predict effects higher than those of the mixture component with the lowest maximal effect, in this case the organochlorine (Figure 5.3, top panel). CA estimates concentrations of mixtures of agents that produce a predetermined effect; and these predictions are based on the effect concentration of each component that will individually produce the same effect. Thus, for mathematical reasons CA predictions for 17β-estradiol: \( o,p' \)-DDT mixtures cannot be made for effect levels above 0.45, i.e. the maximum response obtained in the YES with \( o,p' \)-DDT (Figure 5.1).

![Figure 5.3 - Predictions as given by CA (top panel) and IA (bottom panel) for various molar mixture ratios of E2-\( o,p' \)-DDT based on best-fits to the single agent data (black curves). See legend to Figure 5.2 for details. Molar mixture ratios are 1:5000 (pink), 1:20000 (green), 1:50000 (blue) and 1:100000 (orange).](image)

The CA predictions all plateau at effect levels approaching 0.45. In contrast, the IA model predicted concentration-effect curves that began to plateau at an effect similar to that of 17β-estradiol (Figure 5.3, bottom panel). There were peculiar inflection points
near the median effect concentrations of the predicted curves for mixture ratios of 1:50000 and 1:100000 (17β-estradiol:o,p'-DDT). From the predictions, three mixture ratios were chosen for experimental investigation, namely the 1:20000, 1:50000 and 1:100000 (17β-estradiol:o,p'-DDT) mixtures.

5.2.3 Experimentally observed combination effects: 17β-estradiol-bisphenol A mixtures

The predicted combination effects for the 1:20000 and 1:50000 (17β-estradiol:BPA) mixtures were tested experimentally. Over the entire range of concentrations the observations agreed excellently with the CA predictions (Figure 5.4).

Figure 5.4 - Predicted and observed mixture effects of 1:20000 (top) and 1:50000 (bottom) E2-BPA mixtures. Best-fits to single agent data are shown (black lines) with 95% confidence intervals of mean responses (shading). Predicted effects, computed using the models of CA (red) and lA (orange) are plotted in terms of total mixture concentrations (on the right) and estradiol component of the mixture (on the left). Mixture observations (blue circles) are plotted as for predictions.
There was a complete overlap of the CA predictions with the 95% confidence interval of the best-fit regression line of the observed responses. In both cases the IA predictions were lower than the observations.

As expected, the 1:20000 mixture data show clearly that the presence of BPA in the mixture had an impact upon the effect of 17β-estradiol. When plotted against the 17β-estradiol content of the mixture, the observed responses were considerably higher than those of the hormone alone, and far exceeded the 95% confidence interval of the 17β-estradiol regression line (Figure 5.4, top panel, left set of curves). This modulation of 17β-estradiol by BPA could, of course, also be demonstrated for the 1:50000 mixture (Figure 5.4, bottom panel, left set of curves).

5.2.4 Assessment of 17β-estradiol-o,p'-DDT mixture effects

The observed combination effects of the three tested 17β-estradiol-o,p'-DDT mixtures agreed very well with the lower portions of the CA and IA predictions (Figures 5.5, 5.6 and 5.7).

![Figure 5.5 - Predicted and observed mixture effects of 1:20000 17β-estradiol-o,p'-DDT mixture. Best-fits and 95% confidence intervals of mean responses to the single agents are shown. Observed mixture data (blue circles) and predictions by concentration addition (red curves) and independent action (orange curves) are also plotted in terms of the estradiol component of the mixture.](image)
However, neither of the models were able to cope well with the marked changes in the maximal effects of the mixtures, which decreased as the proportion of \( o,p' \)-DDT in the mixtures rose. Independent of mixture ratio, the CA model predicted a levelling off at effect levels of approximately 0.45, the maximal effect of \( o,p' \)-DDT on its own. The observed responses actually plateau at a level between the maxima of \( 17\beta \)-estradiol and \( o,p' \)-DDT, the precise level of which depended on the mixture ratio (maximal effects of 0.95, 0.72 and 0.61 for the 1:20000, 1:50000 and 1:100000 mixtures, respectively).

Increasing amounts of \( 17\beta \)-estradiol in the mixture also caused increases in the slopes of the observed concentration-response curves, a feature correctly predicted by
CA. It was not possible to confirm experimentally the IA predictions of the maximal effects of the mixtures. Furthermore, the observed concentration-response curves did not show inflection points at median effect levels, as predicted by the IA model.

In summary, there was good agreement between prediction and observation in the low effect range, but neither of the two concepts was able to accurately model the curvatures seen at high effect levels. As was the case with BPA, \( o,p'-DDT \) contributed significantly to the overall mixture effect at mixture ratios of 1:20000 (17\( \beta \)-estradiol: \( o,p'-DDT \)) and higher.

5.2.5 The influence of DMSO on 17\( \beta \)-estradiol-\( o,p'-DDT \) mixture effects

The preceding results led us to hypothesise that one reason for \( o,p'-DDT \) producing low maximal effects may have been its low solubility, coupled with a decreased ability to enter the yeast cells. To test this we included DMSO in the assay medium, at a final concentration of 2%. Previously, DMSO has been shown to lead to marked increases in responses in the YES [Beresford et al., 2000]. However, it was not established whether this was due to better solubilisation of test chemicals or to changes in the properties of the yeast cell wall that enables xenoestrogens to enter and \( \beta \)-galactosidase to leave more easily, or a combination of the two.

As demonstrated by the shift of the 17\( \beta \)-estradiol curve to lower concentrations and the almost 40% increase in maximal response (Figure 5.8), the assay was much more sensitive in the presence of DMSO. The \( o,p'-DDT \) concentration-response curve

![Figure 5.8 - Effect of DMSO in the assay medium on the single agent concentration-response curves.](image-url)
showed a longer linear portion and also gave higher effects; the effect elicited by the highest tested concentration was 1.1 (corrected absorbance units). Due to concerns about solubility, the tested concentrations were not extended into ranges which lead to a levelling off of effects.

The increases in the responses caused by o,p'-DDT individually led to changes in the mixture effect predictions. Most notable was the impact on the CA prediction, which modelled higher responses (Figure 5.9). As with the previous 17β-estradiol-o,p'-DDT mixture responses, there was good agreement between the observations and the CA and IA predictions in the lower portion of the curves. The 1:50000 mixture observations still exceeded the CA prediction at the point where the predicted responses began to plateau. The same was not seen in the 1:100000 mixture, where agreement between CA and observation was good for the entire concentration-response curve.

![Figure 5.9 - Predicted and observed mixture effects of 1:50000 (top) and 1:100000 (bottom) 17β-estradiol:o,p'-DDT mixtures with DMSO in the assay medium. Blue circles are observations, with best fits and 95% confidence intervals being black lines and shading, respectively. CA and IA predictions (red and orange, respectively) are also plotted in terms of the estradiol component of the mixture.](image-url)
5.3 Discussion

Our data demonstrate clearly that the combined effect of 17β-estradiol and BPA or o,p'-DDT is additive. There can be no doubt that weak xenoestrogens such as BPA and o,p'-DDT, when combined with 17β-estradiol, are able to contribute to estrogenic mixture effects. We show that the impact of xenoestrogens on the actions of the steroid hormone depends on the mixture ratio and on its potency relative to 17β-estradiol. When combined at approximately equi-effective concentrations, substantial modulations of the effects of 17β-estradiol by the xenoestrogen became discernible. This implies that the xenoestrogens will contribute to the combination effect at mixture ratios approximately equal to the ratios of the median effect concentrations. Considering that the molar median effect concentrations of BPA and o,p'-DDT are 30000- and 17000-fold higher, respectively, in the YES than those of 17β-estradiol, this should be the case with combinations showing mixture ratios of around 1:30000 (17β-estradiol:BPA) or 1:17000 (17β-estradiol:o,p'-DDT) and above. Our experimental results confirmed decisively these expectations.

The magnitude of effect modulation by the weaker mixture component became apparent when the mixture responses were plotted in terms of hormone concentration, and then compared to the effects of 17β-estradiol on its own. A contribution of the weaker xenoestrogen to the overall combination effect reveals itself as a shift of the mixture concentration-response curves to the left of the 17β-estradiol curve. The observed shifts with the 1:20000 mixtures of 17β-estradiol-BPA and 17β-estradiol-o,p'-DDT were well outside the 95% confidence intervals of the best estimate regression line of the 17β-estradiol concentration-response curves (Figures 5.4 - 5.7, and 5.9). The effects predicted for 1:5000 mixtures, however, did not indicate that combination effects would be distinguishable from those of the hormone alone, within experimental error.

There was generally good agreement between the predictions of the CA and IA concepts and the experimental observations, although interesting deviations occurred. As we stated earlier, it is well established [Greco et al., 1992], that the CA and IA concepts often, but not always, produce differing predictions. There are no generally agreed criteria that would help decide a priori which of the two concepts should be applied to any given endpoint or assay system. For this reason, both concepts were used side-by-
side in the present studies. However, in view of the intrinsic features of the YES it was our expectation that the CA concept should yield the more valid mixture effect predictions. The assay measures the expression of a reporter gene upon activation of the hERα by ligands that bind to the same receptor domain and thus seems ideally suited for the CA concept. The assay is blind to any other effects and precludes the monitoring of dissimilarly acting test agents.

The 17β-estradiol-BPA mixture data confirmed our expectations. With the two tested mixtures the best-fit regression lines for the observed effects were almost congruent with the curves predicted by CA. The IA concept slightly, but systematically, underestimated the observed combination effects for all effect levels (Figure 5.4).

When considering the 17β-estradiol-o,p'-DDT mixture data, a more complicated picture emerged. Again, the CA concept predicted slightly higher combination effects than the IA concept. For low effect levels, the CA predictions overlapped with the 95% confidence interval of the best-fit mean mixture effects, while the IA concept yielded curves displaced to the right of the lower confidence intervals. Furthermore, there were inflection points in the predicted IA curves that did not occur in the experimentally observed concentration-response relationships (Figures 5.5 - 5.7). However, for all mixtures the CA concept failed completely to predict high effect levels and was unable to model the observed maximal effects of the mixtures.

The possible reasons for this system failure deserve serious consideration. We hypothesised that processes related to the low water solubility of o,p'-DDT may have introduced problems with mixture effect predictions. Given that the aqueous solubility limit of o,p'-DDT is 0.35 μM (at 35°C) [Shiu et al., 1990], the actual concentrations of the compound in the culture medium may have been lower than the nominal concentrations used to plot the response curves shown. At some point, further increases in nominal concentrations will have led to disproportionately low rises in the actual concentrations, thus producing the apparent levelling off of the curves seen in Figures 5.5 - 5.7.

A number of reasons lead us to suggest that data on the aqueous solubility of o,p'-DDT alone do not provide sufficient guidance to decide when that point will be reached.

The o,p'-DDT concentration-response relationship exhibited a linear portion. This signifies a range where increases in nominal concentrations are directly related to rises in the biologically effective concentrations of the agent in the culture medium.
Crucially, this occurred at concentrations well above the aqueous solubility limit of o,p'-DDT (Figure 5.1).

Glucose metabolism by yeast cells will cause the production of ethanol during the 72 h period of incubation, which in turn is likely to aid the solubilisation of o,p’-DDT. Transport processes may also work to shift equilibria and facilitate not only solubilisation of the solid material but also increase the rate of secretion of β-galactosidase into the medium. The events following estrogen receptor activation may be subject to further equilibria. Thus, the assay outcome, i.e. colour development in the assay medium, is the net result of a large number of complex dynamic equilibria. The aqueous solubility of the test compound is only one, albeit important, factor in this interplay. It is for this reason that we believe that measurements of actual concentrations of sparingly soluble test compounds alone will not help address the problem. Nevertheless, the systematic deviations between mixture effect prediction and observation we encountered with the 17β-estradiol-o,p'-DDT mixtures seem related to the poor water solubility of the organochlorine.

Our experience with DMSG co-incubations strongly supports these ideas. DMSO acts to increase solubility of the test compounds and may have an impact on the permeability of the yeast’s cell wall, increasing both entry of test compound and secretion of β-galactosidase. As a result, both the linear portion of the o,p'-DDT response curve and the maximal effect increased and this had a positive influence on the predictability of mixture effects. Our experiences show that the predictability of combination effects with mixtures of sparingly soluble agents needs to be carefully explored.

Our results offer guidance in defining conditions where estrogenic chemicals may add substantially to “estrogenic burdens,” over and above the already strong effects of steroidal estrogens. From data about the levels of bioavailable 17β-estradiol and of o,p'-DDT in the serum of post-menopausal women [Deutsche Forschungsgemeinschaft, 1984; Zava et al., 1997] it can be estimated that the molar ratio of the two agents lies between 1:1000 and 1:6000 (17β-estradiol:o,p'-DDT), far below the ratio of median effect concentrations observed in many bioassays. Our data suggest that the contribution of o,p’-DDT alone to combination effects with the hormone are negligible at physiological levels. Similar considerations apply to BPA, although data about human tissue levels are sparse.
However, human tissues contain many compounds with estrogenic activity. On the basis of our studies, it appears conceivable that a multitude of xenoestrogens, when present in sufficient number and concentration, might in principle act together to impact on the actions of steroidal estrogens. Whether such impacts will be physiologically relevant remains to be seen. Definitive answers to this question are currently hampered by our lack of knowledge concerning the full spectrum of estrogenic agents in human tissues. Furthermore, it is necessary to test experimentally whether xenoestrogens are able to act jointly when they are individually present at subthreshold levels (this is a specific aim of the next chapter). Finally, it is at present not possible to assess whether data about the type of joint action (additivity, synergism, etc.) at the molecular level of biological organisation, amenable to study with in vitro assays, are useful for assessment of mixture effects at higher physiological levels.

In summary, our studies, presented in this chapter, do not provide support for the assumption that weak xenoestrogens are generally unable to create an impact upon the already strong effects of endogenous steroidal estrogens. In line with pharmacological principles, they will modulate significantly the actions of steroidal estrogens at mixture ratios similar to the relative potency.
CHAPTER 6 - MIXTURES OF XENOESTROGENS AT INEFFECTIVE CONCENTRATIONS AND THEIR ABILITY TO ENHANCE 17β-ESTRADIOL’S EFFECTS

The findings presented in the previous chapter gave some indication as to the answer to the second of the questions posed in Chapter 4, namely, do weak xenoestrogens contribute to an overall combination effect when the mixture contains the significantly more potent 17β-estradiol? Our studies with binary mixtures of 17β-estradiol and one of the two weak xenoestrogens, bisphenol A and o,p'-DDT, established conclusively that the joint action of a mixture of BPA or o,p'-DDT with E₂ is contributed to by both components. It became clear that, as we had expected, the impact of the weak XE on the effects of E₂ depended upon the relative potency of mixture components and the mixture ratio [Rajapakse et al., 2001].

Mixtures of bisphenol A or o,p'-DDT with E₂ demonstrated substantial modulations of the effects of the hormone when combined at a molar ratio of 1:20000 (E₂:XÉ), approximately equivalent to equi-effective concentrations (EC₅₀). Although these findings confirm the ability of the weaker XEs to contribute to the overall mixture effect, the mixture ratios involved are quite far removed from reality. From available data, it can be estimated that the molar ratios of bioavailable E₂ and o,p'-DDT in the serum of post-menopausal women lie between 1:1000 and 1:6000 (E₂:o,p'-DDT) [Deutsche Forschungsgemeinschaft, 1984; Zava et al., 1997]. In binary mixtures of estradiol and o,p'-DDT, the contribution of the organochlorine to combination effects with the hormone are negligible at these levels.

Human tissues and fluids contain many compounds with estrogenic activity, however, as with o,p'-DDT each agent is usually present at very low concentrations. This laboratory has recently shown that mixtures of eight weak XEs act together to produce significant joint effects when combined at concentrations well below their individual threshold levels [Silva et al., 2001]. These findings, and those of the E₂:XÉ binary mixture studies, raise the possibility that combinations of a large number of xenoestrogens might modulate the effects of 17β-estradiol, even when each individual XE is present at concentrations that alone would not produce measurable effects. Obviously, this possibility has important implications for risk assessment in the field of
endocrine disruption, and we set out to test this idea experimentally. Thus, our overall goal was to investigate the ability of eleven XEs, at individually low concentrations, to modulate the effects of the hormone.

Considerable emphasis was placed on evaluating whether, based on knowledge of the single agents, it was possible to predict mathematically the combination effects of complex mixtures of XEs and E$_2$. The features of the yeast estrogen screen, we believed, favoured the concept of concentration addition because only similarly acting agents will be active. However, in cases with two, three and four agent mixtures, the discrimination between additive combination effects calculated using independent action and concentration addition has not been marked [Payne et al., 2000; Rajapakse et al., 2001]. We now have decisive empirical data supporting our initial belief that concentration addition, and not independent action, is appropriate for use in the YES [Silva et al., 2001]. The concepts of independent action and effect summation led to dramatic underestimations of the experimentally observed response and would have led to the erroneous conclusion of synergistic mixture effects. In the present studies, we chose to utilize the model of CA, which promises the most reliable prediction of expectation on the basis of the potency of individual mixture components. In light of the numerous reports highlighting the inappropriateness of ES in mixture studies where the single agents have non-linear concentration-response curves [Berenbaum, 1985; Kortenkamp and Altenburger, 1998; Borgert et al., 2001], its continued use in exactly such cases [Soto et al., 1995; Arnold et al., 1997; Carlson and Williams, 2001], is alarming. The consequences of its uncritical application cannot, in our eyes, be overstated and its use in these studies was to illustrate these repercussions.

The choice of test agents was somewhat dictated by the use of the reference model of concentration addition. CA estimates concentrations of mixtures that yield a predetermined effect, based on the concentrations of the individual agents that would produce the same effect. Thus, mathematically, CA is unable to predict effects higher that those of the mixture component with the lowest maximal effect. Screening of agents in the YES has shown a number of environmentally relevant xenoestrogens to be only weakly positive, for example $o,p'\text{-DDT}$ and $p,p'\text{-DDT}$ [Payne et al., 2000; Rajapakse et al., 2001]. Their inclusion in complex mixtures of xenoestrogens might have complicated the assessment of agreement between experimental observation and predicted mixture effects. In order to avoid such ambiguities, test agents with high, near maximal, response in the YES (Chapter 4), such as; polychlorinated biphenyls, hydroxylated
polychlorinated biphenyls, phenolic additives and a phytoestrogen, were chosen. Many of these compounds were taken from the study by Miller and colleagues [2001].

It should be restated that the level of exposure to many of the test agents is not known and it is not our aim to demonstrate any more than the application of modelling concepts to complex mixtures of XEs and prove that low levels of XEs can dramatically impact on the effects of 17\(\beta\)-estradiol.

The purposes of our studies made it necessary to estimate as accurately as possible the levels of test agents that did not induce measurable effects when compared with untreated controls. As discussed in Chapter 4, in regulatory toxicology, this task is dealt with by determining so-called No Observed Effect Concentrations (NOECs). However, the hypothesis-testing procedures employed for estimating NOEC values have recently been sharply criticised from a statistical point of view [Moore and Caux, 1997]. It has been shown that these methods all too often lead to considerable underestimations of low effects. To overcome these problems, it has been proposed that it is more appropriate to employ regression-based approaches that estimate low toxic effects by interpolation on the basis of entire dose-response curves with finely spaced data points in the low effect range [Moore and Caux, 1997; Van der Hoeven, 1997]. In fact, we found that testing finely spaced concentrations in the low effect range led to NOEC values that were often below thresholds derived using the regression-based approach (Chapter 4). However, in these studies, we have approximated "insignificant effects" as the concentrations corresponding to 1% of the maximally inducible effect in the yeast estrogen screen (EC\(_{01}\)).

The extensive concentration-response analyses carried out with individual test chemicals in Chapter 4 formed the basis for predictions of entire response curves of defined mixtures of xenoestrogens and 17\(\beta\)-estradiol. A "xenoestrogen pool" was created by combining eleven xenoestrogens (Table 4.1, excluding o,p\(^{\prime}\)-DDT) at ratios proportional to their EC\(_{01}\) values. Based on previous work with binary mixtures that had indicated the ability of XEs to impact on 17\(\beta\)-estradiol (Chapter 5) [Rajapakse et al., 2001], the XE pool was mixed with 17\(\beta\)-estradiol at molar ratios of 1:100000, 1:50000 and 1:25000 (E\(_2\):XE pool) and tested in the YES, using the fixed mixture ratio design described in 4.3 [Altenburger et al., 2000].
6.1 Experimental procedure

Single agent data were obtained exactly as described in Chapter 4. Mixture samples were run in duplicate on at least two separate occasions. Nominal concentrations were used throughout.

6.1.1 Dosimetry

Scattergrams of corrected absorbance values ("effect") versus log concentration were constructed and analysed using the best-fit approach [Grimme et al., 1998]. In these studies we have used the asymmetric Hill function (Eq. (4.1)). The 95% confidence intervals of the best estimate of mean effects were also calculated.

Thresholds are defined as the intersection of the upper 95% confidence limit of the control responses (0 ± 0.018, n = 20), with the lower 95% confidence limit of the best-fit regression model.

6.1.2 Calculation of predicted mixture effects

Following comprehensive concentration-response analysis of the single agents, the responses of mixtures of 17β-estradiol with the XE pool (i.e. all eleven XEs combined according to EC_{01} values, see Table 6.1) were predicted assuming additive combination effects. The expected effects for three mixture ratios were calculated using the models of concentration addition and effect summation. The model of CA has been described in detail in section 5.1.1.

Effect summation simply assumes that the expected effect (E) of a mixture of two agents a and b, equals the sum of the effects of the single compounds at the concentrations present in the mixture;

\[ E_{a+b} = E_a + E_b \]
6.1.3 Assessing mixture predictions

The validity of the predicted mixture effects was evaluated experimentally using 1:100000, 1:50000 and 1:25000 (E₂:XE pool) mixtures. Master stock solutions (1 mM) were made and serially diluted to cover the range of concentrations modelled in the predictions.

The impact of XEs in mixtures with 17β-estradiol becomes discernible when mixture effects are plotted in terms of the hormone’s concentration in the mix, alongside the dose-response curve of E₂ alone. A shift of the mixture dose-response curve to the left of the E₂ curve represents the effect of the XEs. The modulation is considered statistically significant when the 95% confidence intervals for the mixture and E₂ best-fit regressions do not overlap.

6.2 Results

6.2.1 Concentration-response analysis of individual mixture components

All tested agents induced activation of the hERα in a concentration-dependent manner (Chapter 4). Figure 6.1 shows the best-fit regression lines of all the mixture components and 17β-estradiol, where the similarity in shapes and slopes can be seen. However, also clearly evident are considerable variations in potency.

![Figure 6.1](image_url) - Concentration-response curves for the eleven xenoestrogens and 17β-estradiol in the YES. Numbering is as in Table 6.1. The figure shows best-fit regression models (asymmetric Hill function).
Important parameters of the regression models, EC_{01} values and threshold levels are summarised in Tables 4.1 and 6.1. Thresholds are defined as the intersection of the upper 95% confidence limit of the control responses (0 ± 0.018, n = 20), with the lower 95% confidence limit of the best-fit regression model. In all cases, the estimated threshold levels were higher than the corresponding EC_{01} values.

### Table 6.1 - Summary of parameters for test agents in the YES.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Fraction in XE pool</th>
<th>EC_{01} (µM)</th>
<th>Threshold (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 17β-estradiol</td>
<td>-</td>
<td>2.3 x 10^{-5}</td>
<td>3.1 x 10^{-5}</td>
</tr>
<tr>
<td>2. 2',3',4',5'-tetrachloro-4-biphenylol</td>
<td>0.0004</td>
<td>0.008</td>
<td>0.009</td>
</tr>
<tr>
<td>3. 2',5'-dichloro-4-biphenylol</td>
<td>0.0009</td>
<td>0.011</td>
<td>0.012</td>
</tr>
<tr>
<td>4. 4'-chloro-4-biphenylol</td>
<td>0.0054</td>
<td>0.074</td>
<td>0.086</td>
</tr>
<tr>
<td>5. genistein</td>
<td>0.0074</td>
<td>0.086</td>
<td>0.095</td>
</tr>
<tr>
<td>6. 2,4-dihydroxybenzophenone</td>
<td>0.0144</td>
<td>0.124</td>
<td>0.139</td>
</tr>
<tr>
<td>7. benzyl-4-hydroxyparabene</td>
<td>0.0102</td>
<td>0.130</td>
<td>0.148</td>
</tr>
<tr>
<td>8. bisphenol A</td>
<td>0.0554</td>
<td>0.632</td>
<td>0.692</td>
</tr>
<tr>
<td>9. 2,3,4,5-tetrachlorobiphenyl</td>
<td>0.0159</td>
<td>0.177</td>
<td>0.217</td>
</tr>
<tr>
<td>10. resorcinol monobenzoate</td>
<td>0.1950</td>
<td>2.236</td>
<td>2.442</td>
</tr>
<tr>
<td>11. 2,3,4-trichlorobiphenyl</td>
<td>0.1592</td>
<td>2.420</td>
<td>2.797</td>
</tr>
<tr>
<td>12. phenyl salicylate</td>
<td>0.5360</td>
<td>8.824</td>
<td>11.58</td>
</tr>
</tbody>
</table>

* Numbered as in figure 6.1.
* Proportion of each component in "XE pool" before combining with E_2 at ratios described in the text.
* Concentration producing effect 0.017 a.u., i.e. EC_{01} of maximal response in YES.
* Defined as the intersection of the upper 95% confidence limit (CL) of the mean response of controls (0 ± 0.018, n = 20) with the lower 95% CL of the best-fit regression model.

#### 6.2.2 Prediction and testing of mixture effects

To avoid one xenoestrogen contributing disproportionately to the overall combination effect, a pool of xenoestrogens was created by mixing the agents in proportion to their EC_{01} values. In turn, this "XE pool" was combined with 17β-estradiol at mixture ratios of 1:100000, 1:50000 and 1:25000 (E_2:X-E-pool), as we have previously demonstrated that bisphenol A (BPA) could impact significantly on E_2, even when combined at a ratio as low as 1:20000 (E_2:BPA), which approximated to the ratio of their median effect.
concentrations (Chapter 5). On the basis of the relative prevalence of each chemical in the mixture, and on their concentration-response relationships, mixture effect predictions were made using the models of effect summation and concentration addition assuming additive combination effects, and then tested experimentally.

There was very good agreement between predicted and observed combination effects (Figure 6.2), and the combined effect of all the xenoestrogens and 17β-estradiol can be called additive.

![Figure 6.2 - Predicted and observed effects for mixtures of E₂ and eleven XEs. A pool of 11 XEs was combined with E₂ at mixture ratios of 1:100000 (top), 1:50000 (middle) and 1:25000 (bottom), E₂:XE pool. Best-fits to observations (circles) are shown as black curves with 95% confidence bands (shading). CA and ES predictions are the red and orange curves, respectively.](image-url)
As the proportion of 17β-estradiol in the mixture increased, the observed mixture concentration-response curves shifted to lower concentrations (median effect concentrations are 6.2, 4.5 and 3.2 μM for the 1:100000, 1:50000 and 1:25000 E₂:XE pool mixtures, respectively).

In the 1:50000 mixture, there was complete overlap of CA prediction by the 95% CI of the observed mixture data (Figure 6.2, middle panel). However, in the other two mixtures there was only limited overlapping of the very narrow 95% confidence intervals and predictions.

In contrast, the predictions calculated using ES consistently and systematically underestimated the experimentally observed combination effects, independent of effect level. Furthermore, ES was conspicuously unable to model the levelling-off of responses usually seen at high concentrations. Using ES as the assessment model, we would have concluded, erroneously, that the xenoestrogen-estradiol mixtures acted synergistically, because the observed combination effects exceeded those predicted by ES.

6.2.3 Impact of xenoestrogens on the effects of 17β-estradiol

Because of the high potency of 17β-estradiol, it is conceivable that the observed effects of the xenoestrogen-estradiol mixtures were almost entirely due to the action of the steroid hormone. Whether or not this is the case, is not immediately obvious from the plots of mixture effects against the total concentration of all mixture components shown in Figure 6.2. To delineate the effects of the hormone and those contributed by the xenoestrogen pool, we plotted best-fit regression line for the observed responses against the 17β-estradiol content of the mixtures. The resulting curve was then compared to that of the hormone on its own (Figure 6.3). If the xenoestrogens contributed significantly to the total combination effect, increases in response, resulting in displacements of the mixture curves towards lower concentrations would be expected. The extent of this left-shift relative to the 17β-estradiol curve should be more pronounced, the higher the xenoestrogen content of the mixtures.

These expectations were borne out by our experimental observations. With all three mixtures, the contribution of the xenoestrogens revealed itself as a shift of the
mixture concentration-response curves to the left of the 17β-estradiol curve. This shift was most notable in the 1:100000 mixture where the proportion of xenoestrogens was highest. Even with the 1:25000 mixture, the xenoestrogens significantly modulated the action of the steroid hormone, judged by the lack of overlap of the respective 95% confidence intervals of the best-fit regression models.

Figure 6.3 – The impact of xenoestrogens on the effects of 17β-estradiol in the three mixtures: 1:100000 (top), 1:50000 (middle), 1:25000 (bottom), $E_2$:XE pool. Best-fits, with 95% confidence belts, to the mixture data are plotted in terms of the $E_2$ concentrations, alongside the concentration-response curve for $E_2$ alone.
6.2.4 Modulation of the effects of 17β-estradiol by xenoestrogens at "sub-threshold" concentrations

We became interested in visualising the joint effects of xenoestrogen-17β-estradiol mixtures in relation to the responses expected to occur after administration of "sub-threshold" concentrations of each mixture constituent individually. From the data compiled in Table 4.1, the total mixture concentrations resulting from combining all eleven xenoestrogens and 17β-estradiol at their respective NOEC, EC01 or threshold values are 13, 14.7, and 18.2 μM, respectively.

Displayed in Figure 6.4 is the effect of 5 μM of the 1:50000 mixture (top panel)
and 2.8 μM of the 1:25000 mixture (bottom panel) plotted alongside the effects of each agent in the mixture at their corresponding concentrations. Also shown are the combination effect predictions calculated using CA and ES. Each xenoestrogen in the mixture is below its threshold concentration and EC01 value. These two concentrations were chosen as they each lie on the linear portion of the mixture concentration-response curve and are the lowest concentration tested where the mixture and E2 dose-response curves are parallel (Figure 6.3). Both of these concentrations are well below the sum of the NOEC, EC01 or threshold values of the XEs in the mixtures. We can therefore safely assume that none of the xenoestrogens would have produced effects detectable with the yeast estrogen screen when applied singly at these levels. The effect of the 17β-estradiol in the 1:50000 mixture (i.e. 100 pM) is more than doubled by combination with eleven weak xenoestrogens at concentrations individually producing effects below limits of detection. The magnitude of underestimation of observed mixture effects by effect summation is evident in both mixtures.

6.3 Discussion

Perhaps the most important finding of our studies is the clear demonstration that large numbers of weak xenoestrogens are able to modulate the effects of the potent steroidal estrogen 17β-estradiol. This phenomenon occurs even when each individual xenoestrogen is present at levels well below effect thresholds.

Several significant, interconnected conclusions can be drawn from the results of these studies.

(i) The possible negative impacts of xenoestrogens cannot be dismissed as insignificant solely on the basis of their low potency compared with steroidal estrogens. Considered in isolation, the contribution of individual xenoestrogens at the concentrations found in wildlife and human tissues will always be small. However, such reasoning cannot be used to support claims of negligible health risks from weak xenoestrogens, because the number of xenoestrogens present in wildlife and humans is unknown, but likely to be very large.

(ii) Without taking combination effects into account, significant underestimations of the possible hazards associated with exposure to xenoestrogens are inevitable. In our experimental model, we have demonstrated that, in principle, every
xenoestrogen, however weak, adds incrementally to the total estrogentic effect, even at very low concentrations and even in the presence of potent endogenous steroidal estrogens. The implications to hazard and risk assessment are profound. In regulatory toxicology, the focus is on the assessment of single agents [Howdeshell et al., 1999; Tinwell et al., 2000]. Almost by default, the outcome of such exercises is the conclusion that individual estrogentic chemicals pose no risk as they are present at sub-threshold levels in humans and wildlife. Such conclusions have to be called into question in the light of our findings. We show that not even levels below NOEC values can be considered to be without effect, when exposure is to many similarly acting chemicals, each present in small quantities. In such scenarios, the resulting combination effect depends to a large degree on the sheer number of chemicals. It is therefore meaningless to limit the discourse about endocrine disrupters to single agents and their effects. It is now urgently required to take stock of the total estrogentic burden of humans and wildlife, and to undertake a systematic search for endocrine disrupting chemicals in tissues and body fluids.

The data presented in Chapter 5 of bisphenol A-17β-estradiol mixtures may serve to further clarify this somewhat complex issue. Recently, Inoue et al. [2000], determined that the level of BPA in the serum of healthy humans was as high as 0.32 ng/mL (approximately 1.4 nM). Our findings indicated that a mixture ratio of 1:20000 (E2:BPA) would result in a significant modulation of the effect of ivp-estradiol. Using an approximation to serum levels of total endogenous estrogens of 150 pM, levels of BPA in the serum would need to be approximately 3 μM, over 2000-fold higher than conservative estimates of serum levels (i.e. 1.4 nM [Inoue et al., 2000]). Now, in this chapter, 17β-estradiol was combined with a pool of eleven xenoestrogens, in one instance, at a mixture ratio of 1:25000. Each of the XEs in the pool was present at concentrations proportional to their individual EC01 concentrations. Thus, the fraction of BPA in the XE pool was 0.0554 (Table 6.1), which means that the ratio of E2 to BPA in the total mixture was in fact 1:(0.0554 x 25000) or 1:1385. Returning to the serum level of E2 (150 pM), to modulate the effect of the hormone, we now only require a serum level of BPA close to 0.21 μM, still some 150-fold higher than reality. However, what this clearly demonstrates is that by considering BPA as part of a larger pool of only eleven XEs, we have effectively reduced the amount of BPA required to modulate the hormone. Applying this to the real-world situation where exposure is potentially to
hundreds of estrogenic chemicals, it is conceivable that single agent concentrations needed to modulate the effects of 17β-estradiol may reach environmentally relevant levels. The one assumption that has been made is that the agents when combined act additively, which in our studies has clearly been the case.

(iii) The fascination with synergistic effects that has gripped the endocrine disrupter field in the wake of the Arnold paper [1996], is misguided. It has led to a disregard of the significance of seemingly unspectacular additive combination effects. Our results show clearly that additivity is of importance and deserves serious attention in the hazard assessment of similarly acting agents such as chemicals with estrogenic activity.

Much of the discussion about classifying combination effects as “synergistic” (or otherwise) hinges around the formulation of an appropriate expectation of joint effects (the “null-hypothesis”). Many methods exist for the calculation of such mixture effect expectations. Our results demonstrate that simple addition of the effects of individual mixture components, as in the ES method, fails to provide a useful null-hypothesis for the expected effects of xenoestrogen mixtures. Irrespective of mixture ratio and effect level, ES underestimated the combined effects of xenoestrogen and 17β-estradiol. The inadequacies of ES have been discussed previously from a pharmacological point of view [Berenbaum, 1985; Kortenkamp and Altenburger, 1999; Silva et al., 2001]. For some, the insight that responses of single agents of, say, 0.8, 0.9 and 1.1 do not in fact add up to a combined effect of 2.8, appears a hard pill to swallow, but is nevertheless true.

CA yielded quite accurate predictions of the observed joint effects. In the absence of a generally accepted statistical method for assessing agreement between mixture effect prediction and observation, we have used the criterion of overlap between the predicted concentration-response curve and the 95% confidence interval of the best-fit regression model of experimental effects to evaluate the reliability of the CA concept. Figure 6.2 shows that there was considerable overlap with the 1:50000 mixture. Mainly due to the very tight confidence intervals, the predicted curves for the 1:100000 and the 1:25000 mixtures failed to meet the overlap criterion. The displacement of the observed mixture effects to the left of the additive expectation suggests a minor synergism. However, with considerable computing power, it is possible to place a confidence interval around the additive expectation [Grimme et al., 1998], and we feel confident that if this is calculated for these data the two intervals will overlap and
demonstrate that the apparent synergism is in fact additivity. This is in accord with the mechanisms underlying the YES. The assay monitors the events following interaction with the ligand binding domain of the hERα, and is therefore only sensitive to agents that act similarly in the pharmacological sense. In line with the premises of the CA concept [Loewe and Muischnek, 1926], all mixture components contributed to the joint effect in proportion to their individual potencies and their concentrations. Together with earlier observations from our laboratory [Silva et al., 2001], the data presented here confirm the usefulness of CA in predicting fairly accurately the combined effects of xenoestrogens in the YES.

Can our observations concerning the predictability of joint effects of xenoestrogens and their additivity be generalised to include other endpoints relevant to endocrine disruption? The phenomena that ultimately give rise to a color change in the YES culture medium are relatively close to the events following hERα activation. At present we do not know how signal transduction pathways and effector chains responsible for integral effects of estrogenic agents, such as cell proliferation, impact on the predictability of combination effects. Will CA be applicable to integral endpoints? What is the role of other intervening factors, such as absorption, distribution and metabolism? These questions are of practical and theoretical significance, since many pharmacologists regard CA as applicable only to agents that interact exclusively with the same molecular target in a competitive and reversible way [Pösch, 1993]. Using this strict definition of "similar action", interaction of multiple chemicals with the same site does not necessarily imply that the resulting combination effects will follow CA at integral levels of response. Proponents of this view would argue that additional binding to other targets or differing toxicokinetics violate the premises of the CA concept. In these cases, or so the argument goes, the independent action concept should be applied. On the other hand, proponents of the CA concept have claimed that CA represents the "general solution", with IA being a special case of CA [Berenbaum, 1985]. These differences are likely to be settled only by experiments involving side-by-side comparisons of the validity of both concepts in formulating a correct null-hypothesis of expected combination effects. In two elegant studies, Altenburger et al. [2000], and Backhaus et al. [2000], have recently resolved this problem for aquatic toxicants, using algae and bacteria as test organisms. In their hands, multi-component mixtures of strictly similarly acting chemicals (photosystem II inhibitors) followed the CA concept,

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while the effects of mixtures of chemicals with widely varying modes of action were best described by IA.

Another problem concerns the influence of non-estrogenic chemicals on the effects of xenoestrogens. Examples are Ah-receptor agonists such as the ubiquitous polychlorinated dioxins and co-planar polychlorinated biphenyls. It has been shown that these pollutants exhibit anti-estrogenic effects by down-regulation of ER expression [De Vito et al., 1992]. It remains to be seen whether such modifying influences occur at environmentally relevant concentrations of these pollutants.

In conclusion, our results suggest that efforts directed at establishing ineffective levels of individual xenoestrogens are crucially hampered when dealing with mixed exposures. Since each agent contributes to the overall mixture effect even below effect thresholds, the definition of “no effect” exposures is not possible until we know both the number of xenoestrogens we come into contact with, as well as their concentrations in relevant tissues and media.
CHAPTER 7 - CONCLUDING THOUGHTS

Based upon their findings in 1990 [Liehr, 1990; Liehr and Roy, 1990], Roy and Liehr proposed that the carcinogenic and non-carcinogenic adverse properties of estrogens could not be explained solely by estrogen-receptor mediated events [1999]. In terms of the carcinogenicity of estrogens, we agree that estrogen-mediated genotoxicity must be an important contributor. However, we believe that the presence of vast numbers of xenoestrogens in the environment, that interact with the estrogen receptor, may partly explain the prevalence of negative reproductive (non-carcinogenic) effects observed in Man and wildlife by, for example, Sharpe and Skakkebaek [1993] and Colborn and Clements [1992]. In our opinion it is also likely that, via various mechanisms, these xenoestrogens may be implicated in the worldwide increases in breast cancer incidence.

Thus, in this thesis our investigations into the biological effects of estrogens utilised a two-pronged approach. As such, we aimed to determine whether physiological levels of the endogenous hormone 17β-estradiol could in fact induce DNA damage, whilst also investigating the joint action of xenoestrogens via the estrogen receptor.

Epidemiological evidence indicates that breast cancer risk is associated with prolonged exposure to the steroidal hormone 17β-estradiol, resulting predominantly from extended ovarian function [Pike et al., 1993; Toniolo et al., 1995]. There are also numerous reports of steroidal estrogens being carcinogenic in animals [for example, Li et al., 1983].

As yet, however, no animal models have been developed in which tumours are induced by very low doses of E2:

Presumably because of the cost of maintenance of a large number of animals for such a model and the difficulty of dosing in view of the varying levels of endogenous estrogens in cycling females.

Liehr, 2000

The lack of an appropriate animal model may help to explain why the numerous mechanisms proposed over the years by which estrogens are carcinogenic remain, as yet, unconfirmed. Nevertheless, continued in vitro and in vivo studies have served to strengthen one particular hypothesis, the bifunctional pathways to breast cancer [Davis et al., 1998].
The basis of the bifunctional pathways is that estrogens work through genetic and hormonal pathways to increase the risk of developing breast cancer. In the hormonal pathway, 17β-estradiol, its metabolites, and XEs exert their estrogenic or antiestrogenic modulatory effects via receptor-mediated mechanisms leading to aberrant cell proliferation. Demonstrations of the mitogenic properties of 17β-estradiol and XEs are numerous [Payne et al., 2001]. The genetic pathway has also received much investigation and proposes that bioavailable 17β-estradiol undergoes metabolic activation to catechol estrogens that may bind to DNA or undergo redox cycling to produce reactive oxygen species. The hypothesised outcome is that the resulting damage alters the expression of cell cycle related genes, oncogenes, and tumour suppressor genes. It is then conceivable that both mechanisms eventually upregulate aberrant cell proliferation and development of breast cancer.

7.1 The "genotoxic pathway"

The metabolism of 17β-estradiol and the pathway via which estrogens are thought to induce DNA damage were described in detail in Chapter 2. Briefly, various cytochrome P450 isozymes oxidise the steroid hormone to predominantly 16α-hydroxyestrone and 2- and 4-hydroxyestradiol (Figure 2.1). It is the subsequent redox cycling of the catechol estrogens that produces superoxide radicals and leads to the initiation of free radical reactions that are believed to damage the cell's DNA. Of course, this pathway competes with the inactivation pathway involving O-methylation by catechol-o-methyltransferase as well as glucuronidation and sulfation [Martucci and Fishman, 1993].

It was the report by Nutter et al. [1991], describing the detection of DNA single strand breaks following exposure of MCF-7 human breast cancer cells to an estrogen derivative, that stimulated our interest. The metabolism of 4-hydroxyestrone is believed to culminate in the putative production of 3,4-estrone-o-quinone, the derivative investigated by Nutter and colleagues. It occurred to us, that the relevance of this proposed pathway will only be generally accepted when it can be shown that the parent hormone, and not just a putative metabolite, induces DNA damage. Since publication of the original paper, the Comet assay had been developed and theoretically promised to
allow the investigation of whether low concentrations of 17β-estradiol do in fact induce DNA damage.

Following an extensive validation of the Comet assay and its application to the MCF-7 human breast cancer cell line (Chapter 2), we assessed the potential of 17β-estradiol to induce DNA damage (Chapter 3). The validation process proved that, in our hands, results obtained using the Comet assay were highly reproducible. Not only were the results from replicate experiments comparable but, due to its intrinsic analysis of the levels of DNA damage in individual cells, the assay proved highly sensitive.

Our belief that the recently developed Comet assay, offering increased sensitivity, held the key to establishing that 17β-estradiol at concentrations approaching physiological levels induces DNA damage was confirmed. We detected increased levels of DNA damage in MCF-7 cells following exposure to 17β-estradiol at concentrations as low as 10 nM. To our knowledge this is lowest concentration of the parent hormone reported to induce DNA damage and is due, in all likelihood, to the choice of assay system. Further work demonstrated that the DNA damage induced by 17β-estradiol and its catechol estrogen metabolites was not confined to DNA single strand breaks and alkali-labile sites. In fact, using the repair enzyme FPG, the predominant lesion following exposure for only two hours was shown to be oxidised bases (e.g. 8-OHgua). Oxidised bases represent a more sinister proposition to a cell than SSB or ALS, in that 8-OHgua groups are slowly repaired and in rapidly proliferating cells can easily increase critical mutagenic events [Cavalieri et al., 1997].

During the validation period we took the opportunity to assess a technique for data analysis first reported by Bauer et al. [1998], i.e. fitting the tail moment data to the \( \chi^2 \)-distribution (section 2.4.6). It was hoped that this would provide a valid method by which Comet assay data might be appropriately assessed statistically, as reported data analysis methodology is yet to reach consensus, and is therefore often inappropriate. In the case of our positive control, UV-C radiation, the tail moment histograms were modelled encouragingly well by the \( \chi^2 \)-distribution.

Chemical DNA damaging agents, such as hydrogen peroxide or 17β-estradiol, differ from radiation in that a chemical’s damaging capacity is limited by its ability to reach the DNA effectively protected by the DNA’s supercoiled structure. As this does not apply to agents such as radiation, we used the analogy earlier of radiation being rather like a scatter gun, able to induce damage throughout a cell’s DNA. As a result of
this difference, we were surprised to see that in the large proportion of cases involving $E_2$ or $H_2O_2$ the $\chi^2$-distribution fit to the data was very good. However, at very high levels of DNA damage, the tail moment histogram was seen to exhibit a bimodal distribution, clearly not appropriately modelled by a $\chi^2$-distribution. In such populations a higher level of cytotoxicity was detected. This may explain the change in shape of these histograms, as necrotic cells tend to exhibit high tail moments in the Comet assay [McKelvey-Martin et al., 1993].

Thoughtful use of the $\chi^2$-fit to the histograms of tail moments should provide an appropriate means by which to critically appraise Comet assay data. As with all data analysis methodologies, blind application with a lack of understanding of the statistics involved will undoubtedly lead to erroneous conclusions.

### 7.2 Estrogen receptor-mediated effects

Earlier, we alluded to our opinion on the non-carcinogenic adverse effects of estrogens being somewhat different to that of Roy and Liehr’s suggestion that even these effects could not be explained solely by estrogen-receptor mediated events [1999]. In the second part of this thesis (Chapters 4-6), we presented initial work which supports an alternative hypothesis.

The environment contains hundreds of natural and man-made chemicals, albeit at low levels, each capable of interaction with the estrogen receptor. We are not alone in the belief that these xenoestrogens are partly responsible for the reports of deteriorating reproductive health in Man and wildlife. It is also conceivable that these xenoestrogens will play a significant role in the bifunctional pathways to breast cancer and other endocrine-related cancers, in particular the hormonal pathway.

The hypothesis that our data lend support to, proposes that the strong effect of physiological concentrations of 17$\beta$-estradiol can potentially be augmented by these weak xenoestrogens. We believe that although these agents possess weak potency in relation to the steroidal estrogens and are detected in human tissues at low levels, assuming “no risk” is foolhardy. By considering the real situation, in which we are exposed to a multitude of estrogenic agents, an oversight becomes apparent. Single agent estrogenicity studies will only uncover relatively potent chemicals. It is imperative to consider the ability of XEs to act in combination. A natural starting point
is to begin with those XEs with similar modes of action, as used in our studies. Continuing research from this point will perhaps involve mixture studies that include xenoestrogens that act via non-estrogen receptor pathways, before beginning to tackle the role of antiestrogens. What is proposed is a considerable body of work - a long journey - and what we achieved in this thesis is the first of many steps along that path. We demonstrated conclusively the need to consider combination effects and the relevant tools that make the study of such joint action feasible.

The decision to use the yeast estrogen screen for our mixture studies was based on two considerations. Research into endocrine disrupters is primarily concerned with the effects of low doses of these compounds, as environmentally prevalent concentrations are themselves relatively low. The extrapolation of low dose effects from concentration-response data requires a sensitive assay that provides highly reproducible data, and with respect to mixture studies this is even more the case. The concepts utilised to calculate the joint action of chemical mixtures use the parameters of the best-fit regressions to the single agent concentration-response relationships. Unreliable single agent data will impact markedly on the applicability of the joint action predictions. The YES met the requirement for a low inter-experimental variation (Chapter 4) and additionally permitted the design of large-scale experiments such as the twelve component mixture studies (Chapter 6). The second consideration involved levels of complexity, of which we took a pragmatic view. It is wise to make sense of the simplest systems and then apply that knowledge in developing an experimental strategy for the complex multicellular organism. The YES is simple, in that the assay monitors only events immediately following hERα activation, thus avoiding complication by converging signalling pathways and feedback loops. This is probably the reason that the variability is low. Thus, the YES was an obvious choice for us to begin to test our hypothesis.

In Chapter 5 we demonstrated how combinations of 17β-estradiol and bisphenol A or o,p'-DDT act additively, and as such there was no doubt that weak XEs contribute to overall mixture effects. Chapter 6 used these findings as the basis for proceeding to significantly more complex mixtures. In effect we changed the single XE used in the preceding chapter for a “XE pool.” This pool was created by combining eleven different, environmentally relevant, XEs at ratios proportional to their EC90 values and this XE pool was subsequently combined with 17β-estradiol. As with the
binary mixture studies, we were able to demonstrate a considerable modulation of the effects of the potent hormone by the XEs. The significance of these studies became evident when we examined a specific concentration of one of the mixtures (Figure 6.4). At this concentration (5 μM), the effect of each individual XE would have been undetectable and yet when pooled these apparently “ineffective” XEs caused more than a doubling of the effect of the hormone. This is not synergy at work, as demonstrated by the agreement with the concentration addition prediction, this is simply additivity.

Should these initial findings be confirmed in vivo, the significance to epidemiological studies in this field are clear. At present, epidemiologists try to correlate increases in the incidence of adverse health effects with increases in the levels of single agents in serum or adipose tissue [Wolff et al., 1993; van’t Veer et al., 1997]. Our findings suggest that this is likely to be a fruitless task until we know how many endocrine disrupters we are exposed to and more about their mechanisms of action.

7.3 Continuing research

We still stand some way from the certain knowledge of why breast cancer incidence is increasing and what is responsible for the decreases in human male fertility and the increases in reproductive failure in wildlife. However, we believe we have gone some way in demonstrating what is potentially responsible and that the avenues for further research are clear to see.

It must be reiterated here, that although we have shown the ability of 17β-estradiol to induce DNA damage in a human breast cancer cell line, the concentrations were one thousand-fold above physiological levels in premenopausal women. It remains to be seen whether further studies will prove our work to be relevant. However, considered as one component of the hypothesised bifunctional pathway, even a small number of oxidised bases resulting from the redox cycling of estrogen metabolites may be sufficient to result in aberrant cell proliferation, when combined with effects of the hormonal pathway. It cannot be ignored that there is a great potential for the xenoestrogens to also play a role in this pathway. For example, certain XEs compete with 17β-estradiol for plasma protein binding sites [Laidley and Thomas, 2000], thus potentially increasing the free (active) fraction of E₂ in the blood. Also, it is possible that other XEs may alter metabolising enzyme levels [Segura-Aguilar et al., 1997], or be
metabolised to corresponding catechols and may induce DNA damage themselves [Atkinson and Roy, 1995]. There is also evidence suggesting a role for other lipid soluble xenobiotics, such as polycyclic aromatic hydrocarbons and the so-called "food mutagens." Using the Comet assay, Martin et al., have shown human mammary lipid and human breast milk extracts to be genotoxic [1997; 1999], however, the nature of the DNA-damaging chemicals is not known. Our laboratory has preliminary results that an environmentally relevant organochlorine (hexachlorobenzene), induces DNA damage predominantly via reactive oxygen species [unpublished results]. Is it conceivable that relevant levels of endogenous steroidal estrogens, xenoestrogens and non-estrogenic xenobiotics could, via different modes of action, result in detectable levels of DNA damage? It is a distinct possibility.

In terms of proving the relevance of our mixture work, the possibilities for continued work are endless. The most important avenues to explore involve reproducing this modulation effect in mammalian in vitro assays which would provide the opportunity to investigate XEs with differing modes of action. For mixture work of this sort to be carried out in vivo the number of animals required for the construction of the concentration-response relationships may be prohibitive. And yet at present, it is only with in vivo studies that the role of androgens, antiandrogens and antiestrogens might be investigated. Until the effects of antiestrogens can be established many will still believe that the effects of xenoestrogens are likely to be outweighed by the antiestrogens [Safe 1995b], whilst disregarding the fact that antiestrogens can also be deleterious at critical developmental windows.

### 7.4 Conclusion

The potential for the low environmentally prevalent levels of xenoestrogens to act in combination and induce deleterious health effects, based on the results presented here, is great and requires serious consideration. Additionally, relatively low levels of 17β-estradiol induces DNA damage in breast cancer cell lines, a high proportion of which is in the form of potentially mutagenic oxidised bases such as 8-OHgua. These results add further weight to the proposal that estrogens are a risk factor for breast cancer and the bifunctional pathways.
REFERENCES


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