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**Detailed Review Paper
Appraisal of Test Methods for Sex Hormone Disrupting Chemicals**

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Series on Testing and Assessment
No. 21**

Detailed Review Paper

Appraisal of Test Methods for Sex Hormone Disrupting Chemicals

**Environment Directorate
ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT**

**Paris
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The work of the OECD related to chemical safety is carried out in the **Environment, Health and Safety Programme**. As part of its work on chemical testing, the OECD has issued several Council Decisions and Recommendations (the former legally binding on Member countries), as well as numerous Guidance Documents and technical reports. The best known of these publications, the **OECD Test Guidelines**, is a collection of methods used to assess the hazards of chemicals and of chemical preparations. These methods cover tests for physical and chemical properties, effects on human health and wildlife, and accumulation and degradation in the environment. The OECD Test Guidelines are recognised world-wide as the standard reference tool for chemical testing.

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The Environment, Health and Safety Programme co-operates closely with other international organisations. This document was produced within the framework of the Inter-Organisation Programme for the Sound Management of Chemicals (IOMC).

The Inter-Organization Programme for the Sound Management of Chemicals (IOMC) was established in 1995 by UNEP, ILO, FAO, WHO, UNIDO and the OECD (the Participating Organisations), following recommendations made by the 1992 UN Conference on Environment and Development to strengthen co-operation and increase international co-ordination in the field of chemical safety. UNITAR joined the IOMC in 1997 to become the seventh Participating Organisation. The purpose of the IOMC is to promote co-ordination of the policies and activities pursued by the Participating Organisations, jointly or separately, to achieve the sound management of chemicals in relation to human health and the environment.

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PREFACE

The Government of the United Kingdom contributed the Detailed Review Paper (DRP) to the OECD. It was prepared by the UK Medical Research Council's Institute for Environment and Health, on behalf of the UK Department of Environment, Transport and the Regions. It was written by Mr. P. Holmes, Dr. C. Humfrey and Mr. M. Scullion. Additional material was provided by Dr. T. Taylor. A draft form of the report was considered by representatives of OECD Member countries through the forum of the National Test Guideline Co-ordinators. A large number of comments on the draft were provided by Member countries, and have been taken into account in finalising the report. The comments provided support the document as accurate, comprehensive and well balanced and a good basis for the discussion of the development and revision of relevant Test Guidelines. Any recommendations made in the report are those of the authors and do not necessarily reflect those of the OECD.

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FOREWORD

Over a number of years anecdotal observations and scientific evidence have come to light which suggest that chemicals present in the environment may cause adverse health effects in humans and wildlife because of their capacity to mimic or influence the action of endogenous hormones such as oestrogens and androgens.

Current concerns centre around the apparent increase in human and animal disease states linked to the action of sex hormones, and reported observations from human epidemiological studies of reductions in sperm counts and fertility, increases in the incidence of testicular cancer and congenital birth defects in men, and increases in the incidence of breast cancer in women. In wildlife species the occurrence of gross birth deformities, behavioural abnormalities, and both feminisation and masculinisation are major concerns.

Whilst there remains much scientific uncertainty about the nature and magnitude of the human and environmental risks associated with exposure to endocrine-disrupting chemicals, the concerns are such that, in November 1996, OECD Member countries agreed to work together on international projects to:

- co-ordinate national and regional activities concerning the assessment and management of the risk posed by endocrine-disrupting chemicals;
- develop internationally acceptable methods for the hazard characterisation of endocrine-disrupting chemicals; and
- harmonise risk characterisation approaches and regulatory approaches.

This Detailed Review Paper (DRP) is intended make an important contribution to that work by serving as the basis for the first step in the consideration and development of OECD Test Guidelines for the testing of chemicals for endocrine-disrupting effects. OECD Test Guidelines play a critical role in ensuring that efficient and effective procedures are available to identify chemical hazards¹.

The focus of this DRP is on test methods for sex hormone-disrupting chemicals capable of affecting the reproductive process. Other hormone systems which are also important in the control of reproduction, such as the thyroid and adrenal systems, are not considered. In addition, test methods for the effects of sex hormones on non-reproductive processes such as brain development and behaviour are considered to be beyond the scope of this document.

Accordingly, the DRP makes an inventory of existing OECD test methods relevant to the assessment of the effects of sex hormone-disrupters on the reproductive systems of humans and wildlife; describes relevant non-regulatory test methodologies used by the research community; makes a critical assessment of existing test methodologies to detect sex hormone-disrupting chemicals; identifies possible enhancements to existing test methodologies; and identifies both those non-regulatory test methodologies suitable for further development and outstanding research requirements.

1. See "OECD Guidelines for the Testing of Chemicals" and OECD Web site (www.oecd.org/ehs/testguid).

The DRP has been written to provide a state-of-the-art review using literature available up to 1998. As understanding of endocrine disruption and scientific consensus on it continue to rapidly emerge, new tests will be developed which may warrant future consideration and discussion. Concerning existing tests, this report assesses only OECD test methods. Where regulatory authorities in individual countries have their own relevant test methods, it is expected that these will be taken into account when proposals for enhancements of OECD test methods, or for new OECD test methods, are discussed.

Similarly, as scientific consensus on this subject develops, new perspectives and issues will undoubtedly need to be considered. Of particular importance are effects on wildlife. In this area there have been several useful workshops such as the joint OECD/SETAC EMWAT Workshop on Endocrine Modulators and Wildlife Assessment and Testing, held in Veldhoven, the Netherlands, on 10-13 April 1997 (Tattersfield *et al.*, 1997). The report of that workshop provides a useful adjunct to the present text. The Executive Summary is therefore reproduced as Annex 4 to this report for the convenience of readers.

Initially the DRP will be considered in detail by OECD expert groups considering the development and enhancement of Test Guidelines for endocrine disrupters. Taking into account the conclusions and recommendations of the DRP, these expert groups will make further recommendations on priorities and further work on test method development in OECD.

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EXECUTIVE SUMMARY

In recent years several national and international workshops have concluded that chemicals present in the environment may be exerting an adverse effect on human and wildlife reproductive health, and a number of environmental contaminants have shown oestrogenic or related activities in laboratory studies. Although there is as yet no evidence to suggest a causal link between these observations, many gaps in our knowledge have been identified. It has been recognised that there is an urgent need to establish validated *in vivo* and *in vitro* screening assays to test for the oestrogenic and androgenic activities of chemicals.

This report details and critically assesses the ability of existing, relevant OECD test methods to detect a chemical's sex hormone-disrupting potential with regard to reproductive processes. It also reviews a range of non-regulatory model systems that have been used in scientific research, in order to assess their suitability. Consideration of other forms of endocrine disruption (involving other hormonal systems) and of non-reproductive functions of the sex hormones is outside the scope of this document. Potential modifications of or additions to existing regulatory test methods are identified, as are other models considered suitable for formal validation and adoption within an expanded testing battery. Possible approaches to the routine assessment of chemicals for sex hormone-disrupting potential, and gaps in scientific knowledge, are also discussed.

LIMITATIONS OF CURRENT OECD TEST METHODS

None of the existing OECD test methods were specifically designed to detect the endocrine-disruptive activity of chemicals. Indeed, methods for assessing acute toxicity (*e.g.*, in fish, OECD 202; earthworm, OECD 207; or rodent, OECD 401) and those designed to investigate a specific type of toxicity (*e.g.* neurotoxicity, OECD 424) are unlikely to be suited to detecting such processes. Nonetheless, either in their current state or with a degree of modification, some of the vertebrate designs might be expected to be able to detect whether a chemical has significant sex hormone-disrupting activity, although additional studies might then be required to clarify the mechanism of action. Especially in the case of the mammalian studies, several gaps in design can be identified, including the degree of pathological examination of the gonads and secondary sex organs and, for reproductive studies, detailed examination of offspring.

There are very few existing OECD tests in non-vertebrates. Only the reproductive study in daphnids might be expected to be of help in detecting sex hormone-disruption. However, given the different endocrine systems and the wide range of reproductive strategies found among the invertebrate taxa, this test alone would provide insufficient information for hazard assessment. Therefore, there is an urgent need to develop new test models for invertebrates.

POSSIBLE MODIFICATIONS OF EXISTING OECD TEST METHODS

For testing of vertebrates, at least in the short term, the most promising approach seems to be to enhance the existing OECD Test Guidelines. A number of possible enhancements have been identified, many of which are most relevant to the mammalian test designs. These include:

- extension of organ weight and histopathology requirements for gonads and accessory sex organs;
- pathological examination of offspring, where appropriate;
- measurement of sex hormone blood levels;

- detailed assessment of spermatogenesis and/or semen quality;
- monitoring of oestrus cyclicity;
- enhancement of current monitoring of physical and behavioural development, and of learning and memory functions in offspring;
- possibly, investigation of accessory sex organ secretory products.

As noted above, for the study of invertebrate species only the existing test in daphnids might be able to be enhanced to provide more information on sex hormone-disruptive activity.

The sensitivities, and importance as markers of toxic hazard, of the possible endpoints need to be established. Pragmatically, emphasis might first be given to enhancing the subchronic test designs so as to maximise benefit in terms of numbers of chemicals screened. Nonetheless, unless the endpoints used in such designs can be shown to be predictive of adverse effects at all key life stages (*e.g.*, during early development), it will be essential to optimise the sensitivity of existing reproductive study designs and, where appropriate, to develop new models in a range of taxa.

NON-REGULATORY TEST MODELS PROPOSED FOR FURTHER DEVELOPMENT AND ADOPTION

In addition to enhancing existing test designs, it is appropriate to consider the development of other toxicity and reproductive tests in a range of species so as to be able to better assess ecological hazard. This is likely to involve development of a range of multigeneration designs in a range of species, including fish and invertebrates, that could be used for regulatory assessment. Although designs developed by various regulatory agencies throughout the world are likely to be worthy of consideration, it is felt that there will be a need to conduct basic research before an adequate test battery can be developed.

Given the number of chemicals that potentially require testing, there is also a need for a range of simple *in vivo* and *in vitro* models that, even if unable to provide sufficient confidence on which to base regulatory decisions, would nonetheless enable initial screening and prioritisation of chemicals or would be of value in elucidating mechanisms. The following non-regulatory models are proposed for further development, with a view to possible adoption in screening designs:

- the rat vaginotrophic and/or uterotrophic assays to assess potential interference with the oestrogenic hormonal system (there is a need to optimise study designs and perform cross comparisons to determine which are the most suitable for progression);
- the prostate weight of castrated rats as a marker for androgenic hormone modulation;
- assay of vitellogenin in males of oviparous species, which might be a useful biomarker of exposure to oestrogens whilst, as an interim measure, the use of models involving changes in secondary sexual morphology of fish might be appropriate.

Work is also required on developing suitable non-vertebrate models that can detect disruption of endocrine systems having no mammalian correlate, such as the arthropod hormone - ecdysone and juvenile hormone.

At present, it is not possible to recommend the formal adoption of any of the *in vitro* assays because of the various limitations and difficulties inherent in the current designs. These include: *in vitro* endpoints that are dependent on specific receptor or response element interactions, which may not mimic

in vivo modes of action; the inability of many systems to distinguish agonists from antagonists; and the finding that existing *in vitro* models lack satisfactory metabolic systems or may show only limited chemical uptake. Significant interlaboratory differences in specificity, sensitivity and reproductibility also exist for some systems, and the significance of *in vitro* findings must be translated to intact organisms where physiological processes may play critical roles in determining activity. There is also a need to establish the predictability and sensitivity of such models against an appropriate “gold standard” *in vivo* methodology. Further development is recommended of assays using :

- human cell lines, such as Ishikawa and MCF-7 cell lines for oestrogenic activity;
- yeast cells for oestrogenicity and androgenicity;
- the trout hepatocyte vitellogenin assay for oestrogenic activity.

Continued development of structure-activity relationship models is also recommended.

Requirements for Basic Research

A number of general research issues of importance to the understanding of endocrine-disruptive activity have been identified from this review. These include: clarification of basic mechanisms of action of endocrine disrupters (especially the importance of non-nuclear and nuclear receptor-mediated effects and of interactions which are not receptor-mediated; and cross-species differences in pharmacokinetics and pharmacodynamics and clarification of the predictability of effects *in utero/ovo* from data generated using adult forms. Resolution of such questions would be assisted by agreement on a set of reference chemicals and the continued study of structure-activity relationships. There is also a need to rank the relative sensitivity and predictivity of the various endpoints that could be used to assess endocrine disruption, in order to facilitate selection of those endpoints most appropriate for inclusion in regulatory test designs. Basic knowledge of comparative endocrinology needs to be enhanced to facilitate the identification of suitable species (including representative invertebrates) for inclusion in an expanded range of wildlife tests: this is necessary in order to permit adequate screening for endpoints and processes having no mammalian correlate.

With specific reference to the issue of sex hormone-disruption, the following principal research recommendations are made:

- Rank sensitivities of the various marker endpoints, and assess their relevance and relative importance as markers of endocrine-mediated toxicity.
- Establish a reference set of chemicals of defined activity to assist in method development and validation.
- Elucidate the dose-response profiles for endocrine-disruptive mechanisms and apply them to dosage selection during testing.
- Assess whether classical toxicological assumptions of cross-group predictivity hold true for endocrine-disruptive mechanisms.
- Develop simple, inexpensive models spanning a range of ecologically-relevant species, focusing on processes with no mammalian correlate.

- Assess the extent to which interactions occur between sex hormone-disrupting chemicals in mixtures, particularly *in vivo*, and investigate the likely frequency of occurrence of such interactions between different sex hormone-disrupters.

Other more general research needs have also been identified and are discussed in detail in the report.

1. INTRODUCTION

1.1 GENERAL BACKGROUND

1. Over a number of years, anecdotal observations and scientific evidence have suggested that some chemicals present in the environment may have adverse impacts on the reproductive capacity of wildlife species and of man. These have centred around the apparent increase in the incidence of a number of human and animal health effects. For example, human epidemiological studies have suggested effects such as reduced sperm count, reduced fertility, increased incidence of testicular cancer and congenital birth defects in men, and increased incidence of breast cancer in woman. In various wildlife species gross birth deformities, behavioural abnormalities, and cases of feminisation or masculinisation have been reported. Whilst concerns for humans have focused on the potential risk to the individual, in regard to wildlife, the focus has been on the potential impact of such changes on the reproductive success of natural populations and on providing information on which to base ecosystem management strategies. The experimental and observational basis for concerns about endocrine disrupters have been extensively discussed in a series of recent workshops and reports, including:

- *Male Reproductive Health and Environmental Chemicals with Oestrogenic Effects* (Miljøprojekt nr. 290); Report of the Danish Environmental Protection Agency, March 1995 (DEPA, 1995);
- *IEH Assessment on Environmental Oestrogens: Consequences to Human Health and Wildlife (Assessment A1)*; Medical Research Council Institute for Environment and Health (UK), July 1995 (IEH, 1995);
- *Endocrinically active chemicals in the environment (Texte 3/96)*; German Federal Environmental Agency, January 1996;
- “Development of a risk strategy for assessing the ecological risk of endocrine disrupters”; Ankley *et al.*, 1996, as cited in EC, 1997;
- *Research Needs for the Risk Assessment of Health and Environmental effects of Endocrine Disrupters*; United States Environmental Protection Agency, August 1996; and
- *European Workshop on the Impact of Endocrine Disrupters on Human Health and Wildlife: European Commission; 1997.*

2. Although the identified changes could result from a number of toxic mechanisms, there is concern that they may be mediated via disturbance of the normal homeostasis of the endocrine system. Disruption of this key system (*i.e.* endocrine disruption) would have a major impact on an organism, and could show unusual dose-response profiles and markedly greater sensitivity compared with other toxic mechanisms. Thus, although not an endpoint *per se*, endocrine disruption is potentially a highly important mechanism which may necessitate special consideration during the process of a chemical’s hazard and risk assessment.

3. As noted in the Foreword, this review focuses solely on assessing the ability of various test methods and endpoints to detect disruption of the sex hormones (*i.e.* oestrogens and androgens). One of the difficulties currently experienced when considering endocrine disruption is the lack of consensus as to an appropriate definition for the term. Various definitions have been suggested, including those of the US EPA (Kavlock *et al.*, 1996) and the European Workshop (EC, 1997). In this report the approach taken by the European Workshop has been adopted, although the definitions used have been modified to apply specifically to chemical disruption of reproductive processes.

A sex hormone-disrupter is an exogenous substance that causes adverse health effects related to the reproductive function of an intact organism or its progeny, consequent to changes in endocrine function.

A *potential* sex hormone-disrupter is a substance that possesses properties that might be expected to lead to endocrine disruption of the reproductive processes in an intact organism.

4. It is implicit in the definitions above that a chemical can only be definitively considered an endocrine disrupter on the basis of an *in vivo* test model, where a functional endocrine system is present and full interplay between normal physiological and biochemical processes can occur. However, it is accepted that it is possible to identify *potential* endocrine disrupters using other types of model.

5. The following section presents a summary of the objectives of this report, followed by brief overviews of the genetic and hormonal control systems involved in reproduction and the possible mechanisms by which endocrine disruption might occur.

1.2 OBJECTIVES AND SCOPE OF DETAILED REVIEW PAPER

6. The focus of this report is on oestrogenic and androgenic agonistic or antagonistic activity, especially with regard to effects on reproduction. Other forms of endocrine disruption involving other hormonal systems (*e.g.* the thyroid and adrenal) fall outside the remit of the DRP. Thus, the objectives have been to:

- assess the ability of current OECD test methods to identify and characterise the oestrogenic or androgenic-disrupting activities of chemicals;
- identify possible enhancements of existing OECD methods;
- detail and critically assess existing non-regulatory models to identify which, if any, may be suitable for possible formal validation and adoption by the OECD; and
- where gaps in scientific knowledge or in the types of models available are apparent, make suggestions for future research work.

7. Papers on the non-regulatory test models were identified using a structured literature search, supplemented by additional papers referenced in the originally identified papers. Other papers included were either suggested by reviewers of the draft report or obtained through other sources not involving a formal literature search. The models were critically assessed using a number of criteria, including: scientific basis of the method and relevance of endpoints; number and type of chemicals tested; number of workers using the test and consistency of results; development and validation status of the method; test duration and complexity; staff and equipment requirements; cost implications; etc. Further details of the literature search and assessment procedures are presented in Chapter 3.

1.3 SEX DETERMINATION AND REPRODUCTIVE CONTROL SYSTEMS IN THE ANIMAL KINGDOM

8. It is beyond the scope of this report to present a detailed review of the comparative endocrinology of different animal groups, due to the complexity of the systems and the significant

differences that occur between some groups. Here an overview of sex determination and the role of sex steroids in sexual differentiation and reproduction in the animal kingdom is presented. The reader is recommended to consult the literature cited for further details.

1.3.1 Genotypic Sex Determination

9. In most vertebrates and in some invertebrates the sex of an individual is determined by the sex chromosomes at the time of fertilisation, a process referred to as "genotypic sex determination" (GSD). In animals exhibiting GSD, a sexual maturation control system is established which directs and controls all of the late ontogenetic processes involved in male-female differentiation of the genitalia. However, this genetic determination is not final and irrevocable; many external and internal environmental factors may come into operation during the developmental process which could modify or completely reverse the phenotypic expression of the genetic constitution of the individual. In some groups the importance of environmental influences is great: for example, the effect of temperature on sex determination in some reptiles. The fundamental principles of sex determination in animals have been reviewed by many authors (*e.g.* Turner and Bagnara, 1971; Pieau *et al.*, 1994; Fry, 1995).

10. The two major types of existing genotypic sex determination may be referred to as the mammalian and avian types, also denoted as the XX/XY and ZZ/ZW types, respectively. In animals exhibiting the XX/XY system, the male is the heterogametic sex (XY) whilst the female is homogametic (XX). Half of the spermatozoa produced contains an X-chromosome and the remainder the Y-chromosome. In the ZZ/ZW system of sex determination, the female is the heterogametic sex. The small chromosome (equivalent to the Y-chromosome of mammals) is designated by the letter W, and the X-chromosome equivalent by the letter Z. Thus the homozygous (ZZ) condition produces males, whilst the heterozygous (ZW) produces females with half the ova carrying a W-chromosome and the other half a Z-chromosome. All the sperm carry a Z-chromosome (reviewed in Turner and Bagnara; Pieau *et al.*, and Fry). However, in some species it seems that the situation is actually more complex than in the two simple model systems described above. Recent research suggests that in some cases autosomal genes can have a role in sex determination (see review by Wachtel *et al.*, 1991). In contrast, the sex of some poikilothermic vertebrate groups is determined solely by environmental temperature, whilst in others there appears to be an interaction between environmental temperature and the genetic system (Pieau *et al.*). Sex determination in vertebrates is summarised in [Table 1](#).

1.3.2 Sex Differentiation and Hormonal Control In Mammals

11. In mammals, the early fetus displays internal duct structures which would allow it to develop into either a male (the Wolffian ducts) or a female (the Müllerian ducts). Sexual differentiation of the female is generally largely autonomous and is regarded as the default pathway of fetal development. Thus the Müllerian ducts normally persist and the Wolffian ducts regress automatically unless hormonal signals (initiated in the genotypic male) dictate otherwise. Development of the fetus into a male requires, initially, the formation of the testes (containing Sertoli cells) and, subsequently, the production of hormonal signals which stimulate masculinisation of the body. The formation of a testis containing Sertoli cells is dependent upon expression of the *Sry* gene on the Y-chromosome. The Sertoli cells then begin to secrete anti-Müllerian hormone (AMH), which causes regression of the Müllerian ducts (which would otherwise give rise to much of the internal female reproductive tract). Soon after testicular differentiation, Leydig cells form within the testis and begin to secrete testosterone. This acts locally to stabilise the Wolffian duct system, thus preventing regression. Testosterone is also secreted into the systemic circulation and acts at multiple sites around the body to bring about full masculinisation.

Table 1: Sex determination in vertebrates²

Taxon (Class, order, suborder)	Genotypic sex determination (GSD)		Temperature-dependent sex determination (TSD)
	XX/XY	ZZ/ZW	
Mammals	✓		
Birds		✓	
Reptiles			
Crocodilians			✓
Turtles	✓	✓	✓
Squamates			
Lizards	✓	✓	✓
Snakes		✓	
Amphibians			
Anurans	✓	✓	✓
Urodeles	✓	✓	✓
Fish			
Teleosts	✓	✓	✓

XX/XY, male heterogamety; ZZ/ZW, female heterogamety

12. Before its action at peripheral target sites, testosterone is converted into the more potent androgen, dihydrotestosterone (DHT), by the enzyme 5α -reductase. Deficiencies in 5α -reductase, AMH or testosterone production, or in the actions of testosterone or DHT, can result in various intersex conditions in genotypic males. Conversely, overproduction of androgens (usually as a result of malfunction of the adrenal glands) may result in the partial masculinisation of a genotypic female. In addition to these hormonal actions, it is increasingly being recognised that several other hormones play subsequent roles in the development of a normally functioning male testis and reproductive tract. These include the regulation of Sertoli cell number by the action of follicle-stimulating hormone (FSH) and the thyroid hormones. Emerging evidence suggests that oestrogens and inhibins also play an important, but as yet unspecified, role in the development of the normal male reproductive tract.

13. The developed male reproductive system is controlled by complex interactions of the endocrine system. In the hypothalamus, neuroendocrine neurones secrete gonadotropin-releasing hormone (GnRH) into the hypophyseal portal system, where it is carried via the blood to the anterior pituitary. Here it stimulates the release of the gonadotrophic hormones, luteinising hormone (LH) and follicle-stimulating hormone (FSH). Prolactin is also released from the anterior pituitary under the control of dopamine. The hypothalamic neuroendocrine neurones contain receptors for monoamines and are therefore affected by these CNS transmitters. The target of action for LH and FSH is the testis. Although the primary actions of each hormone are on different testicular cells, increasing evidence suggests that their function and

2. Adapted from Pieau *et. al.* (1994)

specificity may be more complex than was previously thought. The function of prolactin is less clear. It appears that it may potentiate the effect of LH in the testis.

14. In the female, a complex series of interrelated steps are under hormonal control via the hypothalamo-pituitary axis. These steps involve follicular development, ovulation, ova transfer, fertilisation, transport of the conceptus, and its subsequent implantation and development culminating in parturition. As is the case with the male, gonadotrophin-releasing hormones are released from the hypothalamus and are carried via the hypothalamo-hypophyseal portal system to the anterior pituitary, where they promote the release of follicle-stimulating hormone (FSH), luteinising hormone (LH), prolactin and others. The target organ for FSH and LH is the ovary. The role of prolactin is again less clear. Depending on the species studied, it is thought to have effects on the corpus luteum, luteolysis and lactation. The release of GnRH from the hypothalamus is controlled by a number of feedback loops, mainly involving hormones released by the ovary, oestrogen and progesterone. Although not fully understood, it is postulated that follicular growth under the influence of FSH promotes the secretion of increasing amounts of sex steroids which in turn have a negative feedback effect on the hypothalamic pathways, resulting in decreased levels of FSH from the pituitary.

15. These processes are described in further detail in Brook and Marshall (1996) and Griffin and Ojeda (1996).

1.3.3 Sex Differentiation and Hormonal Control in Non-Mammalian Vertebrates

Birds

16. Sex differentiation in birds was reviewed by Fry (1995). The initial organisational events within the gonad are similar in birds and mammals. Development of the ovarian architecture occurs when oestradiol is synthesised by the gonad, causing localisation of the primordial germ cells in the cortex of the left ovary rather than the default location of the seminiferous tubules, as occurs in males. In addition, the release of oestradiol causes regression of the right gonad and suppression of the synthesis of Müllerian regression factor - the glycoprotein which, in the absence of oestrogen, causes regression of the developing oviducts in males. Thus oestradiol is responsible for retention of the left Müllerian duct and for its differentiation into the functional left oviduct and shell gland. Studies indicate that steroid hormone production by the chick gonad begins between day 2 and day 6 of incubation. It is highly likely that such hormones are involved in the differentiation of the gonad itself, as well as determining the differentiation of the accessory sex organs.

Reptiles and Amphibians

17. Many crocodylian- and turtle-type reptiles exhibit temperature-determined sexual development. The genetic factors associated with sex determination in reptiles, and thus with gonadal development, remain to be precisely resolved, but the role of temperature and the influence of exogenous exposure to sex steroids has been extensively studied. In alligators the incubation temperature at specific critical periods of embryonic development is known to trigger the determination of sex. Incubation temperature induces an all-or-nothing response, so that an embryo will be either male or female; few intersexes are produced. In the red-eared slider turtle (*Trachemys scripta*) a warm temperature (e.g. 31°C) will produce all-female hatchlings, a cooler temperature (e.g. 26°C) will produce all males, and intermediate temperatures will result in varying ratios of males to females. The influence of environmental temperature has, however, been shown to be modifiable by treating eggs with oestrogens or oestrogen analogues. Studies have shown that alligators and several species of turtle can exhibit sex reversal (male

to female) if developing embryos are exposed to an oestrogenic compound during a specific period of development (Guillette *et. al.*, 1995).

18. In some amphibians low temperatures during larval development are known to result in the differentiation of predominantly females, whilst at higher temperatures males predominate. Exogenous chemicals are also known to be implicated in sex reversal phenomena; the African frog *Xenopus* has been extensively used in experiments on sex reversal. Young larvae raised in water containing oestradiol display reversion of genetic males to fully functional females. The exposure must occur at a critical period of development and need not continue for more than three days. In contrast, exposure to exogenous androgens does not appear to interfere with normal gonadal development in females (Guillette *et. al.*).

Fish

19. In terms of sex determination, fish species exhibit GSD (XX/XY and ZZ/ZW) and also temperature-dependent determination (Pieau *et. al.*, 1994). Reproductive processes are known to be significantly more varied among bony fishes than in any other vertebrate class, with some species becoming sexually mature as males and later developing naturally into females, or vice versa. On an evolutionary scale the more primitive the group, the less evidence there is for control of the reproductive cycle (Scott, 1987). In terms of phylogeny, the cyclostomes are the most primitive group and the teleosts the most recent. Gonad development in teleosts is completely controlled by the pituitary, which is linked to the hypothalamus via direct innervation and a blood portal system. Gonad development in the elasmobranchs appears to be only partially dependent on the pituitary, and the hypothalamus and pituitary are not intimately linked. In the case of the cyclostomes, gonad development appears to be autonomous and the pituitary and hypothalamus are physically separated by a cartilaginous barrier.

20. In teleosts the steroid hormones are involved in the differentiation of the gonads and accessory ducts. Many studies have shown that exogenously applied steroid hormones can bring about complete reversal of sex. It is also known that fish gonads develop a capacity for sex steroid synthesis very early in development. For example, the histologically undifferentiated gonads of first-feeding rainbow trout fry (50 days after hatching) can synthesise progesterone, 17 α -hydroxypregnenolone and 17 α -hydroxyprogesterone. For detailed reviews of the reproductive endocrinology of fish, see Scott.

1.3.2 Sex Determination and Differentiation and Hormonal Control in Invertebrates

21. Invertebrates exhibit both asexual and sexual reproduction, with 99% of species reproducing sexually at some point in their life cycle (Barnes *et al.*, 1993). Asexual reproduction takes the form of fission or parthenogenesis; in the latter, diploid eggs are produced without fusion of gametes. In species exhibiting parthenogenesis, the shift from parthenogenetic to sexual reproduction is often brought about by changing environmental conditions. Related to parthenogenesis is the process of arrhenotoky, in which unfertilised haploid eggs develop into males and fertilised diploid eggs develop into females.

22. Some invertebrate taxa always have separate sexes assigned to separate individuals (gonochorism), whilst in others individuals have both male and female organs at the same time (simultaneous hermaphroditism) or may lose the characteristics of one sex and gain those of the other, or may even alternate their sex during their lifetime (sequential hermaphroditism).

23. According to Barnes *et. al.*, there are basically three types of sex determination in invertebrates: maternal, genotypic and environmental. However, these influences can be interactive. In *maternal*

determination, the female is able to produce different types of eggs (the sexual destiny of which is predetermined) or to control whether eggs are fertilised or not, which in turn dictates the sex of the offspring. An example of an organism exhibiting the former type of maternal sex determination is the minute polychaete, *Dinophilus gyrociliatus*, although male and female sperm are also known to occur, with female sperm consistently selecting large eggs and male sperm consistently selecting small eggs. Species of Hymenoptera, for example the honey bee (*Apis mellifera*), exhibit the second type. Queen honey bees can determine whether their eggs are fertilised or not; fertilised eggs develop into females whilst unfertilised eggs develop parthenogenetically into males (diplohaploid sex determination).

24. *Genotypic* sex determination is found in species of insects, arachnids, crustaceans, nematodes and the polychaete *D. gyrociliatus* (Barnes *et. al.*, 1993; Charniaux-Cotton and Payen, 1988). The principles of genotypic sex determination basically follow those of genotypic sex determination in vertebrates.

25. *Environmental* conditions are also known to determine the sexual development of the embryo or larva in some invertebrate species. For example, in the echiuran worm *Bonella viridis*, planktonic larvae become female if they settle on the surface of mud, whilst those settling on or very close to an existing female become male. In the slipper limpet (*Crepidula fornicata*) sex determination can be influenced by social conditions. These molluscs arrange themselves into stacks, and as the stack grows the sex of those individuals in the stack is determined by their position and their size: the lowermost individuals are female; the uppermost individuals are males; and those in an intermediate position are often hermaphrodite (see Barnes *et. al.*). Size-specific sex change responses have also been shown to be controlled by local group composition in the shelf limpet (*Crepidula norrisiarum*) (Warner *et al.*, 1996). Natural populations of polychaetes of the genus *Capitella* contain males, females and hermaphrodites. Heterogametic individuals are females, whilst the sex of homogametic individuals is labile. Laboratory investigations show that the number of individuals living together and the sex ratio affects the initial sex of homogametic individuals: *en masse* they are initially male and can become simultaneous hermaphrodites, whilst on their own they develop as females and may occasionally become hermaphrodites (Petraitis, 1990). Photoperiod is known to influence sex determination in some crustaceans, for example in the amphipod *Gammarus duebeni* (Watt, 1994). In the protogynous intertidal isopod *Gnorimosphaeroma luteum* it has been shown that females are more dominant in the spring and early summer compared to late summer and the autumn, when immature males are dominant (Brook *et al.*, 1993). Infections are also known to affect sex determination in invertebrates: for example, feminisation has been well documented in *Gammarus duebeni* infected by a microsporidan parasite (Dunn *et al.*, 1995).

26. Further details on sex determination and differentiation, and on reproduction and hormonal control of reproductive processes in selected invertebrate taxa, are presented below.

Annelida

27. In annelids, the majority of the available information on the endocrine control of gametogenesis relates to female polychaetes. This reflects the ease with which oocyte development can be studied in these animals by measurement of cell diameters. In most polychaetes, oocytes are shed into the coelom at an early stage of development, where growth and maturation are completed. More rarely the oocytes remain in contact with the germinal epithelium, forming discrete gonads attached to the peritoneal wall.

28. In many polychaetes the body form changes to a distinctive reproductive state, termed an "epitoke", at the approach of sexual maturity. The reproductive form arises from the asexual animal by either metamorphosis or budding. In general, maturation of eggs and sperm and transformation to the

reproductive epitoke are controlled by one or more brain neurohormones. Removal of the brain from a young worm will result in a rapid process of maturation, forming a precocious epitokal metamorphosis which is abnormal and incomplete. Normal reproductive development appears to depend upon the gradual withdrawal of brain neurohormones with increasing age. These hormones are thus inhibitory at high concentrations, but appear to be necessary at low concentrations if the reproductive changes are to occur normally (see Highnam and Hill, 1969; Kershaw, 1984).

Mollusca

29. The phylum Mollusca presents a bewildering variety of physical forms and a number of different reproductive strategies and processes. In a few of these there is evidence for neurosecretory control of reproductive processes, but the low level of hormones in the animal and the poor understanding of the biochemical aspects of reproduction still represent a major problem in understanding the processes. Gastropods tend to be hermaphrodite, although this is by no means the rule. In contrast, the bivalves and the highly developed cephalopods have separate male and female sexes. Much of the recent work on molluscs has centred around the occurrence of imposex in response to exposure to organotin compounds (*e.g.*, tributyltin, TBT). It has been shown that sexual differentiation in males is mediated by testosterone-like androgens. The occurrence of imposex, in which females are partially masculinised, is thought to be caused by inhibition of aromatase activity; this enzyme is responsible for the metabolism and resulting detoxification of androgens. Despite this work, much still has to be learnt regarding the biochemical mechanisms which control reproductive processes in molluscs.

Crustacea

30. Decapod crustaceans such as crabs, shrimps, lobsters and crayfish have well developed endocrine systems that are second only to those of the insects in complexity among the invertebrates. Hormones are known to play an important role in phenotypic sex determination in crustaceans. Young genetic males develop an androgenic gland associated with the genital tract. This gland, which does not develop in genetic females, masculinises the differentiating gonads into testes and also induces male secondary sexual characteristics. Implantation of an androgenic gland into a young genetic female causes transformation of the ovaries into testes. Thus, as in mammals, femaleness is the default pathway of sexual development and the crustacean reproductive system becomes male only if provided with an appropriate hormonal signal. Absence of this signal results in a female form. Crustacea, like insects, must periodically moult their exoskeleton in order to grow and reach a sexually mature form. Unlike insect exoskeletons, those of most crustacea are heavily calcified. This difference in exoskeletal composition is associated with some differences in the hormonal control mechanisms of moulting. The control process is very much in keeping with that of insects, but with one major difference. In crustaceans the neurosecretory control of steroid secretion by the ecdysial glands is inhibitory. This is in contrast to the excitatory neurosecretory control of the prothoracic glands in insects.

Insecta

31. Insects generally reproduce by sexual processes, although asexual forms of reproduction are not uncommon, frequently playing a very important role in the normal lifecycle (*e.g.* this is common in many aphids). It was at one time believed that the morphological differences between male and female insects were strictly genetically determined, and the discovery of intersexual mosaics provided good evidence for this view. It is now known that in at least one species of glow-worm, *Lampyris noctiluca*, both the primary and secondary male characteristics are induced by an "androgenic" hormone secreted from paired endocrine glands associated with the testis. Transplantation of these glands into genotypic female larvae

induces masculinisation. In the insects, hormones generally play permissive roles in the development of the reproductive system but are not normally involved in sex determination of reproductive structures. The major reproductive role of hormones is to guide normal development in the egg. Juvenile hormone is necessary for egg development, inducing the synthesis of vitellogenin for the eggs. It may also act on the ovary to promote vitellogenin uptake and oocyte maturation. However, spermatogenesis in most species apparently proceeds without juvenile hormone.

32. The development of adult genitalia is a major characteristic of insect metamorphosis and moulting behaviour. Thus, in a limited sense, the combination of hormones which control the metamorphosis and moulting processes may also be said to influence reproduction. As insects grow, they are compelled periodically to shed their exoskeleton and synthesise a new, larger one; this process of moulting is precipitated by steroid hormones called ecdysteroids, which are secreted by the prothoracic glands. At each moult neural processes activate the secretion of a hormone, prothoracicotrophic hormone (PTTH), which travels through the blood to the prothoracic glands. They, in turn, secrete an ecdysteroid, ecdysone. This hormone is peripherally activated to the ecdysteroid, 20-hydroxyecdysone, which acts on target cells in a manner similar to that of steroids in vertebrates. The steroid binds to intracellular receptors, inducing transcription of specific genes. Juvenile hormone is a terpenoid and plays a key role in the moulting process, being responsible for the retention of larval characteristics. The absence of juvenile hormone during the final moult triggers the maturation to the adult (sexual) form. In many species this involves pupa formation and metamorphosis steps.

MECHANISMS OF SEX HORMONE-DISRUPTION

33. The mechanisms of action of many agents suspected of affecting one or more aspects of reproductive function have yet to be fully determined. For a given chemical, they may involve multiple sites of action and complex disturbances in the homeostatic processes.

34. Sex hormone-disrupters and their metabolites may, if structurally similar to endogenous ligands, interact directly with the physiological ligand's receptor in the cells of the gonads or accessory sex organs. This may mimic the action of the hormone, resulting in receptor stimulation and resultant biological effects (agonistic), or may block or reduce the binding and biological activity of naturally present hormones (antagonistic). An alternative mode of action would be to modify post-receptor signalling pathways within cells, whilst effects might also result from alterations in higher levels of organisation within the body, for example interference in the hypothalamo-pituitary-gonadal axis in the brain-pituitary-thyroid axis, or in neurotransmitters in the central nervous system. Indirect effects could also result from the chemical-inducing or -inhibiting metabolic enzymes, causing changes in the production or breakdown of endogenous hormones or alterations in carrier proteins in the blood. A chemical may be directly active or may act indirectly, requiring initial metabolic activation. Thus disruption of endocrine secretion, binding, feedback control or target activity can be affected by action at several sites. When an alteration in hormone activity is observed, it is often not clear whether this is the result of a primary effect on hormone secretion and on subsequent receptor interaction or whether the effect is a response to organ damage or some other mechanism.

35. Other factors that must be addressed include consideration of the species exposed, as the roles of specific steroids vary between species and basic physiological and sexual differentiation processes can also differ significantly. The developmental stage at which exposure occurs is also particularly important. Effects of exposures at a critically sensitive period of the life cycle (*e.g.* during embryonic development) have been termed "organisational effects" because they may lead to permanent structural modification of various body systems. Whilst these effects can be manifest in early life, their impact may not be felt until

later, possibly not until the adult phase. When exposed during adulthood, the effects on the endocrine system are more commonly transitory and have been termed “activational effects”.

2. OVERVIEW OF EXISTING REGULATORY TEST METHODS

2.1 REGULATORY BACKGROUND

36. World-wide a complex array of test designs is used to characterise the potential toxic hazard of a chemical for subsequent risk assessment processes. For a novel chemical the test requirements will depend upon many factors, including: the economic region in which the chemical is to be marketed, its intended use (*e.g.* agrochemical, non-agricultural pesticide, food additive/supplement, industrial product, chemical intermediate/by-product, or pharmaceutical); the quantities marketed (usually assessed on a tonne per year basis); the class and state of scientific understanding of chemicals with similar structure; physico-chemical parameters; method of manufacture (*e.g.* the regulatory requirements for chemicals produced using biotechnological processes may be different from those for chemicals produced by a conventional chemical manufacturing process); and intended usage pattern (*e.g.* test requirements for pharmaceuticals depend on target condition, treatment regimen and number of treatments during life, etc.). A large number of existing (mainly industrial) chemicals which have been continuously manufactured over several decades have been accepted as safe in the absence of overt toxic effects in humans or exposed wildlife during this period (*i.e.* a “generally recognised as safe” (GRAS) approach). In the case of other chemicals, additional testing or full re-registration may have been performed or may be under consideration.

37. Different approaches have been developed in various countries or economic areas/communities, resulting in complex and potentially contradictory requirements for toxicity testing. It is beyond the scope of this report to present a comprehensive review of all the test designs which have been approved for regulatory purposes, nor is it appropriate to review here the various risk assessment strategies currently applied. However, in recent years there has been growing pressure to consider toxic endpoints (including sex hormone-disruption) that were not previously of concern and to develop a more internationally harmonised approach to testing.

2.2 OECD ACTIVITIES

38. The OECD produces internationally recognised guidelines on test methods for investigating the health and environmental effects of chemicals that can be used in providing data needed in carrying out hazard assessment for regulatory processes. A wide range of OECD Test Guidelines concern *in vivo* toxicity assessment, including a number that specifically address reproductive aspects. In addition, there are some *in vitro* methods for investigating mutagenic potential. There is an established updating procedure for revision of existing OECD guidelines, or the introduction of new ones, based on advances in science or animal welfare considerations³.

39. A number of the existing OECD guidelines are relevant to the issue of sex hormone-disruption and are listed below in [Table 2](#). In view of increasing interest in chemicals that may act as endocrine disrupters, it is appropriate to consider whether the existing guidelines are adequate to address this particular aspect or require enhancement. An alternative or supplementary strategy might be to validate promising novel methods. In order to identify possible enhancements of existing guidelines, the endpoints have been summarised in [Annex 1](#). As part of its ongoing review process, the OECD Test Guidelines Programme has endorsed draft versions of some of the relevant methods.

3. See “OECD Guidelines for the Testing of Chemicals” and OECD Web site (www.oecd.org/ehs/testguid).

Table 2: List of Relevant OECD Test Guidelines

OECD	Study type	Adopted
202	<i>Daphnia</i> species: acute immobilisation test and reproduction test	02/04/84
203	Fish, acute toxicity test (Updated Guideline)	17/07/92
204	Fish, prolonged toxicity test: 14-day study	04/04/84
205	Avian dietary toxicity test	04/04/84
206	Avian reproduction test	04/04/84
207	Earthworm, acute toxicity test	04/04/84
210	Fish early life stage toxicity test	17/07/92
401	Acute oral toxicity	24/02/87
407	Repeated dose 28-day oral toxicity study in rodents	27/07/95
408	Repeated Dose 90-day Oral Toxicity Study in Rodents	Updated 21/9/98
409	Repeated Dose 90-day Oral Toxicity Study in non-Rodents.	Updated 21/9/98
412	Repeated dose inhalation toxicity: 28/14-day	12/05/81
414	Prenatal Developmental Toxicity Study	Updated 22/01/01
415	One-generation reproduction toxicity	26/05/83
416	Two-generation reproduction toxicity	Updated 22/01/01
420	Acute oral toxicity – fixed dose method	17/07/92 #
421	Reproduction/development toxicity screening test	27/07/95
422	Combined repeated dose toxicity study with the reproduction/developmental toxicity screening test	22/03/96
423	Acute oral toxicity - acute toxic class method	22/03/96 #
424	Neurotoxicity study in rodents	21/07/97
451	Carcinogenicity studies	12/05/81
452	Chronic toxicity studies	12/05/81
453	Combined chronic toxicity/carcinogenicity studies	12/05/81
478	Genetic toxicology: rodent dominant lethal test	04/04/84

A number of other relevant OECD guidelines also exist. However, details are not presented here because they only differ significantly from one of the above guidelines in respect of route of administration (*e.g.* OECD 410: Repeated dose dermal toxicity study: 21/28-day, has similar endpoints to OECD 412: Repeated dose inhalation toxicity study: 28/14-day).

Revised Draft Guidelines for OECD TG 420 and 423 are in development (October 2000).

2.3 REVIEW OF RELEVANT OECD TEST GUIDELINES

40. The endpoints examined by OECD Test Guidelines are quite variable. This is unsurprising given the Guidelines' different principal objectives. None of these tests was specifically designed to detect endocrine disruption. They are used as general screens for various adverse effects. Nonetheless, either in their current state or with some modification the tests could be expected to detect an adverse response if a chemical had significant endocrine-disrupting activity, although further tests to clarify the action of the chemical and the mechanisms operating would then be required. The Test Guidelines may be grouped into the following major categories for review.

2.3.1 Mammalian Study Designs

Mammalian acute toxicity (OECD 401, 420, 423)

41. These simple tests, generally performed in the rat, have limited endpoints because they were designed only to provide estimates of the acute (single dose) toxicity of a chemical. Given that some effects of sex hormone-disrupters are known to develop rapidly following a single dose of a chemical (*e.g.* oestrogen agonists and antagonists and uterine/vaginal effects), it might at first sight be thought appropriate to incorporate a number of simple endpoints with limited impact on cost and resources (for example, oestrous cycle monitoring and organ weight analysis). Although it would also be possible to include other, more complex endpoints (such as histopathological examination and detailed sperm analysis), the associated cost would certainly be excessive in relation to the test's principle objective. The inclusion of any additional endpoints should also be approached with caution for this type of study, as in the acute tests animals are liable either to be moribund or to show frank toxicity. Hence interpretation of derived data would be very difficult. It must also be recognised that significant numbers of such tests are conducted mainly in one sex.

Mammalian subchronic toxicity (OECD 407, 408, 409, 412)

42. These studies have been designed to assess a wide range of toxic endpoints under conditions of subchronic administration to rodents or non-rodents, as appropriate. They include measurement of multiple biochemical, haematological, pathological and organ weight endpoints, in addition to a general monitoring of behaviour, appearance and growth. When the current versions are considered, it can be seen that there is considerable scope for inclusion of additional endpoints of relevance to sex hormone-disruption. In particular, the range of secondary sex organs routinely examined is rather restrictive. The proposed revisions to OECD 408 and 409 are therefore to be welcomed, because the additional examinations would considerably strengthen the test design. Consideration should be given to expanding the text to give advice on suitable approaches for monitoring/interpreting hormonal measurements, and to a similar enhancement of the remaining Guidelines in this category. A number of other possible additions are indicated in Table 2, but the potential benefits of incorporation of additional requirements must be weighed against the cost and increased complexity of the study design.

Neurotoxicity study in rodents (OECD 424)

43. This Guideline is unusual in that the focus is on one particular aspect of adverse effect, *i.e.* neurotoxicity. In addition, the duration and study requirements may vary considerably depending upon the particular chemical and stage of development, with durations of between 28 days and over than one year

being within the scope of the Guideline. Given the highly focused nature of the test, it seems unlikely that inclusion of additional endpoints relating to sex hormone-disruption would be appropriate.

Mammalian long-term (OECD 451, 452, 453)

44. In these tests animals are administered test chemicals for prolonged periods at levels up to the maximum tolerated dosage. In the case of rodents, the treatment period will cover the majority of their life span. The question of the impact of prenatal and neonatal exposure on test sensitivity is briefly addressed in OECD 451 and OECD 453, but may warrant further consideration in the light of current scientific knowledge.

45. All studies require assessment of general behaviour, growth pattern and appearance. In chronic toxicity studies detailed biochemical and haematological profiling is included. However, it is noteworthy that the optional inclusion of additional tests such as hormonal assays (as specified in the subchronic tests) is not addressed; this aspect may warrant review. In the case of the carcinogenicity investigations, where the principle endpoint under consideration is neoplasia, the requirements for clinical pathological assessment are obviously much more limited.

46. Currently there is only a requirement to weigh gonads, but not accessory sex organs, in the chronic toxicity studies. Possible extension to include the accessory organs should be considered. The absence of weight recording from the carcinogenicity test is justifiable, given the wide disparity in weights occurring in geriatric animals and the consequent problems in data interpretation. All these test methods already require extensive histopathological examination of both the gonads and accessory sex organs. A possible enhancement might, however, be the inclusion of guidance on appropriate histological processing techniques to maximise the information derived.

47. Because of the tests' chronic nature, they offer the possibility of assessing the effects of chemicals on sex organ neoplasia and, potentially, on reproductive life span. Detailed monitoring of changes in reproductive capability with age would, however, necessitate considerable modification of the design of the studies or institution of a separate test methodology. Nonetheless, inclusion of oestrus cycle monitoring and enhanced assessment of spermatogenesis, together with greater focus on reproductive organ status during histopathological examination in the chronic tests, may offer useful initial information and act as an alert to potential problems.

Mammalian teratogenicity (OECD 414)

48. This study design is intended to assess the teratogenic potential of a chemical by treatment of pregnant females for a short period during gestation. In addition to teratogenic change, endpoints of potential significance to an assessment of sex hormone-disruptive action include alterations in the size, sexual differentiation and sex ratio of the fetuses and in abortion levels and embryonic and fetal death. Potentially the degree of pathological examination of the dams could be increased, but because these aspects are more intensively studied in the one- or two-generation studies (see below) the benefit of inclusion would be questionable.

Mammalian reproductive function (OECD 415, 416, 421, 422)

49. These studies are intended to provide information, of varying levels of completeness and complexity, on a chemicals' effects on the general reproductive performance of a rodent. The simplest design, OECD 421, is intended to act as an initial chemical screen or as a preliminary range-setting

exercise, to be followed by a more rigorous investigation. The range of endpoints is, of necessity, restricted in this study design by the short study duration, as animals are generally killed on day 4 post-partum. This restricts the range of endpoints that can be investigated in the pups and also limits assessment of maternal behaviour. Many endpoints of value in assessing potential sex hormone-disruption are nonetheless included (see Table 2). Possible additional endpoints that could be considered for inclusion without significantly altering the character of the test include: gonadal and accessory sex organ weight analysis, monitoring of secretory product production, sex hormone level assay, detailed spermatogenesis and sperm analysis, and, in pups, a more rigorous pathological examination. The scope of the combined toxicity and reproductive/ developmental test, OECD 422, is similar in nature to that of OECD 421, except that some additional endpoints are indicted as possible options (e.g. hormone levels, *corpra lutea* count). OECD 415 could be extended in a similar manner to OECD 421 and might benefit significantly from an extension of the physical and behavioural developmental monitoring of the pups and from assessment of memory and learning performance because they are currently permitted to survive to weaning.

50. The two-generation reproductive toxicity test, OECD 416, is rightly regarded as the most rigorous of the current OECD mammalian reproductive tests. Unlike the other two designs, it permits in-depth study of the growth, development and sexual functionality of the F_1 generation and includes the monitoring of the subsequent (F_2) generation through to weaning. In the current design a number of potential enhancements can be identified. However, the majority of these have been addressed in the revised version currently awaiting approval (see Table 2). The only areas that might still be worthy of consideration are physical, sexual and behavioural development and the learning and memory abilities of offspring, inclusion of hormonal level monitoring, and possibly investigations into the secretory products of the accessory sex organs.

Mammalian dominant lethal (OECD 478)

51. The dominant lethal assay is intended to detect mutagenic effects on the development of the gamete. In the OECD rodent design, males receive either single or repeated doses of test material before mating with untreated females. During gestation the female is killed and pre- and post-implantation losses are assessed. Questions may be raised as to the interpretation of the data derived from single dose studies because of the possibility of missing particularly susceptible stages in gametogenesis. However, potentially this type of study will permit assessment of mutational events during spermatogenesis. In the "Guidelines for Reproductive Toxicity Risk Assessment," US EPA (1996), reference is made to an alternative design in which effects on oogenesis are assessed using treated females.

52. Non-mutagenic mechanisms may also affect the endpoints studied in the dominant lethal test. These include effects upon sperm number, failure of sperm transport, or ovum penetration or alteration in mating behaviour. The endpoints of potential significance to detection of sex hormone-disrupting potential in the current study design include time to mating, the mating behaviour of the males, and an assessment of fecundity and pup loss. Potential additions to the test methodology include pathological examination of the treated animals, monitoring of accessory sex organ secretory product production and hormone levels, and detailed examination of sperm and spermiogenesis. Given the currently non-routine nature of this test, it is unlikely to be of value include such parameters routinely unless previous data indicate a potential need.

2.3.2 Non-Mammalian Study Designs

53. The majority of the current OECD biotic Guidelines call for a simple assessment of the acute to subchronic toxicity of a chemical in various classes of animals. The endpoints are limited to mortality and changes in gross appearance or behaviour. The fish acute and subchronic toxicity (OECD 203 and 204), avian dietary toxicity (OECD 205) and annelid acute toxicity (OECD 207) Guidelines are examples. It seems unlikely that additional endpoints could usefully be included in these designs, particularly as the animals used either are immature forms or may be too small for recommendation of routine inclusion of detailed physical examination for changes in reproductive organs or hormonal or other assays.

54. Existing OECD non-mammalian Guidelines that include endpoints of potential value in assessing sex hormone-disruptive action are thus limited to those detailed below. The ability of the tests to predict the sex hormone-disruptive potential of a chemical throughout an ecosystem is clearly open to question and will be discussed further later.

Avian reproductive function (OECD 206)

55. In this test, birds are fed the test chemical for at least 20 weeks, during which egg laying activity is stimulated by control of photoperiod. Eggs are then artificially incubated and the young maintained and observed for a further 14 days. This method includes a wide range of endpoints of relevance to sex hormone-disruption. They include, for the adult: survival, condition and behaviour; rate of egg production and viability levels; egg shell thickness and number of cracked eggs; and gross pathology of the gonads and accessory sex organs. Possible additional endpoints that might be considered for inclusion are: organ weight analysis; histopathology examination of sex organs and tissues; and hormone assay. Endpoints assessed for the hatchlings are restricted to number and survival of young; condition; growth; and general behaviour. Possible enhancements include organ weight and pathological examination of the chicks. Although potentially revealing adverse effects in the offspring, the long-term maintenance of the young to permit assessment of their subsequent growth and behavioural and sexual development (including sex hormone levels) would constitute a significant change in the nature of the study because it would then become closer to the mammalian multigenerational protocols. This cannot currently be justified as a routine approach.

Fish early life stage (OECD 210)

56. Effects of chemical exposure on the development of the early life stages of fish are investigated in this study design. A number of endpoints are used to assess these stages of development. It is considered unlikely that additional endpoints could be included without a significant extension of the duration and nature of the test to include the subsequent growth and development of the young.

Daphnid acute and reproductive function (OECD 202)

57. This design includes an assessment of the effect of chemical exposure on daphnids over a period of at least two weeks. It assesses changes in mortality, behaviour and reproductive capacity over a number of generations and therefore will act as a screen for potential adverse hormonal or other toxic activities, although mechanistic information will not be provided.

2.4 POTENTIAL ENHANCEMENTS OF EXISTING OECD TEST GUIDELINES

58. Several endpoints have been identified as worthy of consideration for inclusion in the existing OECD Test Guidelines. However, it is recommended that for each study type the relative benefit to be gained from inclusion of any possible addition first be established and that optimised sampling regimens and conditions be determined. Because it is likely to take some time to investigate these aspects for the full range of mammalian studies, it is recommended that attention first be focused on refining the subchronic test designs rather than those relating to chronic toxicity or the longer duration reproductive studies. This would improve as rapidly as possible the screening for endocrine disruptive activity of the largest number of chemicals, because subchronic tests would tend to be used on more chemicals. In addition, as the subchronic tests are generally conducted early in a chemical's development sequence, early detection of potential activity would enable appropriate modifications to be made to subsequent studies to assist in hazard evaluation.

2.4.1 Mammalian Species

59. In summary, additional endpoints that could be considered for inclusion are:

a) Vaginal cytology

This is known to be dependent upon the hormonal balance of the female and to respond rapidly to the administration of chemical possessing: for example, oestrogenic agonist or antagonist activity. Thus the inclusion of an assessment of oestrus cyclicity by examination of vaginal smears or washes offers a quick and easy way to measure the sex hormone status within the female and is of value in interpreting of other findings (for example, weight or pathological data for the female reproductive organs). Potentially this technique could also act as a simple initial marker of changing reproductive capacity with age in chronic studies.

b) Sperm development

In some cases detailed assessment of spermatogenesis can offer significant insight into the functionality of the male testis, which can be affected by a variety of mechanisms including hormonal disturbance. However, as advised in the 1996 US EPA Guidelines for reproductive toxicity risk assessment, careful processing of the tissue is required to maximise the information obtained. Guidance on suitable techniques would therefore be necessary in the test methods. Similarly, sperm morphology, number or activity assessment could be of potential value. Samples can be obtained by ejaculation or from the vas deferens, epididymus or testis, depending on the species under consideration and the requirements. However, assessment and interpretation may be difficult and subject to variation. This area is still under development, with interest being shown in the development of computer-assisted semen analysis (CASA) systems to automate the analytical process. The daily sperm production (homogenisation-resistant testicular sperm and total caudal sperm counts) in the young adult offspring has also been suggested as a sensitive marker (EC, 1997). Such approaches offer potential benefits, but it has been suggested (Jahn and Gunzel, 1997; Creasy, 1997) that pathological examination of the gonads may be preferable.

c) Organ weight analysis and pathological examination

Extending the existing provision for organ weight analysis of the gonads and accessory sex organs in parental animals and offspring may be useful. The weights of many of these organs are known to be very dependent on appropriate hormonal stimulation. Hence any change may be due to a disturbance in sex hormone homeostatic mechanisms, although other mechanisms could result in similar changes. Evidence of an effect would, however, only indicate the need for subsequent clarification of mode of action and significance. Similarly, greater use of gross and histopathological examination of reproductive organs may provide valuable information on the reproductive and hormonal status of an animal. It is considered that provision of guidance on optimal methods for the conduct of histological processing and examination of the sex organs may also assist in standardisation and improvement of assessments.

d) Hormonal monitoring

Increasingly the technology exists to accurately assay blood hormone levels in a range of common laboratory species. A number of the existing Guidelines refer to the optional inclusion of hormonal assays, although there is no further clarification in the existing text and sex hormones are not specifically identified as of concern. Monitoring of sex hormones in animals may be of potential benefit in detecting a sex hormone-disrupting agent. However, it must be remembered that hormone levels are not static but show diurnal and other rhythms and vary with stage of development. Repeated, carefully controlled sampling protocols would therefore be required in order to establish whether there was a true effect. Indeed, it may be that measurement of multiple hormones and consideration of their relative ratios would be necessary to gain a full picture. The endocrine system is a highly complex homeostatic system, so that only transitory fluctuations may be seen in blood levels after initial exposure and these are then rapidly masked by compensatory changes. These compensatory changes, although not detectable from blood hormonal monitoring, may in time result in long-term harm to the animal.

e) Accessory sex organ products

The products of the accessory sex organs play a key role in reproduction. An assessment of the functionality of these organs could prove of value. These aspects have not been widely addressed in toxicological assessments to date, but should be considered for possible future development and validation.

f) Behavioural modification

Stress should be placed on monitoring the mating behaviour of treated animals and, where appropriate, that of their offspring because subtle changes in such behaviour patterns arising from chemical toxicity could have profound consequences. Similarly, the pattern of maternal behaviour will be a key factor in the survival, growth and socialisation of any offspring. Consideration should be given to developing and validating standardised, robust assessment techniques. Subtle effects of chemicals can also be shown by assessment of the learning and memory functions of offspring exposed *in utero* or postnatally.

g) Physical development

The physical development of offspring of exposed parents is a sensitive marker for adverse effects of a chemical. As previously noted with reference to parental animals, gonadal and accessory sex organ weight recording can provide valuable information, whilst a number of

other endpoints relating to sexual development have been well established in a variety of regulatory guidelines and in the scientific literature. These include, in males, day of preputial separation and nipple development, and, in females, day of vaginal opening, onset of oestrus, and regularity of oestrus cycling. A number of other markers are also available, including intersex, other genital abnormalities, anogenital distance at birth, and, potentially, the size of the pre-optic region of the brain.

2.4.2 Non-Mammalian Species

60. The scope of the current OECD Test Guidelines for non-mammalian wildlife species is very limited. In particular, clear reproductive endpoints are only assessed in daphnids and avian studies and, to a more limited extent, in the fish early life stage test. Given the variations in endocrine system and reproductive strategy found in different taxa, it can be appreciated that the current range of studies is inadequate to assess a chemical's potential ecological hazard. There is thus an urgent need to extend testing to cover a wider range of species and endpoints to improve predictivity. Possible additions to the current test models have nonetheless been identified, including the increased application of organ weight analysis, pathological examination of the sex and accessory sex hormones, and blood hormone assays. In addition, it is generally accepted that, for a chemical showing toxic potential, detailed information on its fate (including the potential for bioaccumulation in offspring and, in the case of mammals, in milk) is of great value in assessing the potential risk posed by exposure. Clearly, provision of such information would be of particular relevance where chemicals are suspected of having sex hormone-disrupting activity.

61. This review has identified possible modifications to the existing OECD Test Guidelines that could enhance their ability to detect endocrine-disrupting activity ([Annex 1](#)). Particularly in the case of the short-duration test designs, which might be useful as early screens, consideration should first be given to assessing the relative benefit of including additional endpoints in the existing guidelines as opposed to introducing simple "stand alone" tests focusing solely on the detection of sex hormone-disrupting activity. In any case, before additional endpoints are adopted, their ability to detect endocrine effects should be ranked, so as to determine which offer the greatest benefit.

62. Some other OECD Test Guidelines are in early draft form, *i.e.* still under development. These are:

- OECD 211: *Daphnia magna* reproduction test;
- OECD 212: Fish, short-term toxicity test on embryo and sac-fry stages; and
- OECD fish, juvenile growth test (in development).

63. Test Guideline 211 is intended to improve upon the output from the OECD 202 daphnid reproduction test. Possible additional endpoints that could be considered during test development are limited to monitoring of parents for abnormal behaviour/appearance, perhaps including an option to extend monitoring of reproductive performance to subsequent generations. Both fish tests focus on particular stages of development. Draft OECD 212 is intended to act as a bridge between lethal and sublethal tests, to act as a screen before a full early life stage test (OECD 210), or to be used on species where husbandry techniques are insufficiently developed to allow prolonged maintenance. Given these intended roles and the range of endpoints already included in the draft, it is unlikely that further additions are warranted. The juvenile development test is intended to provide information on the effects of

prolonged exposure to chemicals (28 days) on juvenile animals with, as currently conceived, only growth (using weight) and gross appearance/behaviour being monitored. Within these limitations, it is considered unlikely that minor modifications to the design would provide information of relevance to assessing sex hormone-disruption.

Other existing tests

64. Consideration should also be given to reviewing existing test designs developed elsewhere than in the OECD, because these may include or suggest approaches of potential value. Examples include the fish partial and whole life models of the US EPA and the designs arising from the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) and the EMWAT report (Tattersfied *et al.*, 1997). The reader is also referred to the European Centre for Ecotoxicology and Toxicology of Chemicals Compendium of Test Methods (ECETOC, 1996), which reviewed several non-OECD regulatory methods and endpoints (see Table 3). However, as mentioned later, it is apparent that development of novel tests will be necessary to provide sufficient coverage of the range of endocrine and reproductive systems found in different taxa.

Table 3**Study types included in ECETOC, 1996**

Study type	Regulatory body	Animals used
Subchronic toxicity		Rats, mice, dogs
Chronic toxicity	EPA, JMAFF, OECD	Rats, mice, dogs
Multigeneration reproduction And/or continuous breeding	EPA, FDA, JMAFF, OECD	Rats, mice
Reproductive and developmental toxicity	ICH	Rats, mice, rabbit
Prenatal developmental toxicity	EPA, FDA, JMAFF, OECD	Rats, mice, rabbit
Reproduction/developmental toxicity screen	OECD 421	Rats
Combined repeated dose toxicity with reproduction/developmental toxicity screen	Draft OECD 422	Rats
Dominant lethal test	EPA, OECD	Rats, mice
Early life stage	OECD 210	Fish
Partial chronic test	EPA	Fish
Full lifecycle	EPA	Fish
Invertebrate reproduction	ASTM, OECD	Daphnids, chironomids, mysid shrimps and annelids
Avian reproductive study	EPA, OECD	Mallard duck, quail

ASTM American Society for Testing and Materials
EPA US Environmental Protection Agency
FDA US Food and Drug Administration
ICH International Congress on Harmonisation
JMAFF Japanese Ministry of Agriculture, Forestry and Fisheries

3. CRITICAL ASSESSMENT OF NON-REGULATORY TEST METHODS

3.1 INTRODUCTION

65. Non-regulatory test methods of potential relevance to sex hormone-disruption were identified by literature searches in a number of databases (Medline, Toxline, Embase, Biosis, CA Search, Pascal, Environline, Pollution Abstracts and CAB Abstracts). The structured searches were completed in August 1996, using the terms detailed below. However, additional papers were used which had been referenced in the papers found in the original search, suggested by reviewers of the draft version of this report, or identified from other sources.

Literature search terms

Use of :

(O)estrogenic, androgenic, anti(o)estrogenic, antiandrogenic,
(o)estrogen-agonist, (o)estrogen-antagonist, androgen-agonist or
androgen-antagonist

In same sentence as:

Response(s), activit(y/ies), effect(s), properties or potenc(y/ies)

with reference also mentioning:

test, assay, bioassay, screen, radioimmunoassay, immunoassay,
biomarker or biological marker

66. The test methods identified are described in detail in Annex. They have been assessed and compared based upon a number of criteria, including:

- relevance of method and endpoints to sex hormone-disruption (*i.e.* scientific basis in light of known mechanisms);
- reported specificity, sensitivity and consistency of test;
- reported number and type of chemicals tested;
- development/validation status of method;
- number of workers reporting on the test model;
- test duration and complexity, staff/equipment requirements and cost implications;
- patent status of test method; and
- for *in vivo* tests, numbers and types of animals required.

67. The results of the critical assessment are summarised below. For convenience, these are grouped under the following categories:

- *In vivo* models relevant to assessment of potential toxicity to humans.
- *In vitro* models relevant to assessment of potential toxicity to humans.
- *In vivo* models relevant to assessment of effects on wildlife.
- *In vitro* models relevant to assessment of effects on wildlife.

68. Only tests considered of little or no relevance to humans were included in the “wildlife” categories. However a number of the tests in the “human-relevant” category could also be predictive of effects in mammalian or vertebrate wildlife species.

3.2. Relevant *in vivo* methods for potential effects in humans

3.2.1 Non-Specific Tests

Rodent Teratology Screen

69. Chernoff and Kavlock (1982) proposed the adoption of an abbreviated *in vivo* teratology study protocol as an initial screening tool to assess teratogenic potential. In this assay, pregnant mice were only dosed on days 8 to 12 of pregnancy and were then maintained and permitted to give birth. The endpoints assessed were restricted to pup number and weight on days 1 and 3 after birth. As a validation exercise, over 25 chemicals with widely differing structures and activities were investigated. The presence or absence of teratogenicity had previously been investigated in regulatory-compliant teratogenicity studies on the chemicals.

70. The result reported indicated a reasonable degree of predictivity of teratogenic potential by the screen when compared to the reference studies. However, the assay proposed had very restrictive endpoints and used a main study with large groups (24 to 30 dams/group). Considering the numbers of animals required, the extensive study duration (several weeks), the limited and non-specific endpoints and the lack of corroborative data from other laboratories, the use of this model is questionable. Certainly it does not constitute a suitable screen for sex hormone-disruptive activity. Should a rapid teratogenicity screen be needed for use in the selection or prioritisation of chemicals, it would be more appropriate to develop an *in vitro* fetal incubation model because this would be likely to prove more efficient in terms of time, cost and use of animals.

Rodent Pregnancy Maintenance Tests

71. Phillips *et al.* (1990) and Kunhnz and Beier (1994) used similar methods to study the effects of progesterone, norethindrone, ethynyl oestradiol, norgestimate and levonorgesterol on the ability of ovariectomised rats to maintain pregnancy. The basic method involves ovariectomy of pregnant females followed by administration of the test chemical and assessment the chemical’s ability to maintain pregnancy. As such it constitutes a method for identifying progestational activity.

72. The data presented indicated that the method was generally effective. However, there are concerns about dosage responsiveness, with levels of pregnancy maintenance possibly being inhibited at high dosages of some of the chemicals. Thus, when used as a screen with little information available on

which to base dosages, it is possible that the outcome could be misinterpreted depending on the dosages used. Given these concerns, and the requirement for surgery, the adoption of this test model as a routine initial screen cannot be supported.

Rat Implantation Assay

73. Work by Dahr and Mattu (1995) on two potential contraceptive agents involved assessing their anti-implantation activity in rats. Females were treated from shortly after mating, and implantation sites counted on day 11 of pregnancy. The endpoint is very restricted, but could be influenced by several mechanisms of chemical action. Given that significantly more information is generated by current teratology protocols, no further consideration of this test is warranted.

Rodent Vaginal Assay

74. A modified version of a rodent vaginal assay (see below) was proposed by Deckers and Schuurs (1989) to detect aromatase inhibition. This model was based upon treating hypophysectomised rats with an oestrogen precursor and monitoring the resultant changes in vaginal cytology when chemicals suspected of having aromatase-inhibiting activity were co-administered. Any inhibitory action would block production of oestrogen from its precursor and thereby prevent changes in vaginal cytology.

75. The ability of this hypophysectomised rat model to detect aromatase inhibition was established using a small number of chemicals, but the method is open to several criticisms. The surgical preparation of animals may be criticised as technically demanding and stressful, and could result in modifications to normal physiology and metabolic profile which could impact upon data interpretation. An alternative chemical hypophysectomy process addresses in part the technical and stress reservations, but the problems inherent in the maintenance of hypophysectomised animals still exist. In addition, the test system has only been used for a limited number of chemicals and has not been widely validated. Given the restricted endpoint and questions on reproducibility, predictability and practicality, it is not considered suitable for use in a routine screening battery. The model's potential for use in detailed mechanistic investigations is, however, recognised.

Rodent Behavioural Modification

76. Because of the close relationship to the mouse fetal exposure model the studies on mating behaviour in adult hamsters and rats are assessed below under oestrogen-related tests.

3.2.2 Oestrogen-Related Tests

Ovariectomised Primate Models

77. Models were developed by Schane *et al.* (1972) and Paliwal *et al.* (1992) in which oestrogenic agonistic and antagonistic activities were assessed using ovariectomised rhesus monkeys. The endpoints used to assess the activity of a number of oestrogens, including oestradiol, in the Schane *et al.* model were perianal sexual-skin colour and the onset of menstrual-like bleeding following treatment withdrawal. Paliwal *et al.* used a similar animal model to study the effects of two oestrogen antagonists on fallopian tube proteins.

78. The use of a non-human primate would be anticipated to facilitate extrapolation to humans. The method of Schane *et al.* provides simple endpoints and the possibility to re-use animals, whilst the Paliwal

et al. model was more complex and had less clearly defined endpoints. Both protocols would, however, require extensive testing against a wide range of chemicals. In particular, it would be necessary to assess the sensitivity to chemicals with only low oestrogenic potential and the response to others with known potential to disrupt other, associated hormonal systems. Nonetheless, it must be recognised that in many countries the use of primates is severely restricted because of ethical and legal constraints, particularly where surgical preparation is required. Financial and logistical aspects may also restrict their availability. Given that suitable alternative systems exist, it is impractical to consider the use of such a model as a routine screening test.

79. As an aside, should it prove essential to study aspects of a specific chemical's interaction with the oestrogen system in a non-human primate, the availability and expense of the rhesus monkey is itself problematic. Under such circumstances, it may be advisable to consider establishing a model based upon a more readily available non-human primate.

Rodent Uterine Assays

80. The rodent uterotrophic models, and the rodent vaginotrophic response, have been widely used over many decades as bioassays for a wide range of oestrogenic and anti-oestrogenic agents. With this popularity has come considerable diversity in study design and endpoints used, such that for both the uterotrophic and vaginotrophic models there cannot at present be said to be a definitive and generally accepted protocol design.

81. Both rats and mice have been widely used in the uterotrophic model. There is little evidence of significant differences in overall sensitivity between the two animals although the effects of a particular chemical have been shown to vary between different species. The mouse may possibly offer a high degree of sensitivity and is easier to handle and more economical to maintain. However, the rat is the animal most widely used in routine regulatory toxicology and more information may therefore be expected to be available on dosimetry, pharmacokinetic, pharmacodynamic and toxicity aspects. This would be expected to assist in dosage selection and in interpreting the significance of findings. Overall, it is considered that the rat would be preferred as the default rodent for this assay. The work of Phillips *et al.*, 1990 suggests that the rabbit may be a suitable non-rodent model in those instances where rodents prove unsuitable.

82. As previously discussed, there has been considerable divergence in the protocols used. Some models involve testing immature animals that have not yet begun natural oestrus cycling. This approach has the disadvantage of requiring tight control of the schedule for using a given batch of animals. Other models, including the test battery approach of O'Connor *et al.* (1996), have used pre-study ovariectomy to overcome this time limitation and to ensure a consistent response by the animals. Clearly, ovariectomy involves some additional effort and cost and necessitates the development of appropriate surgical expertise (this is likely to be particularly evident in the case of mice). The surgery also represents some additional stress to the animals. In a review of the structure-activity relationships of oestrogens, Jordan *et al.* (1985) considered the available test methods for estimation of oestrogenicity. They considered that, for *in vivo* uterine growth models, ovariectomy of animals has proved an unnecessary precaution given the consistent responsiveness found with immature animals. In view of these differing opinions, inclusion of ovariectomy in an initial screening model is open to question.

83. Several endpoints have been used in the various test designs. The majority of studies have included uterine weight as a major endpoint. However, even here there is considerable divergence in the exact definition: for example, inclusion/exclusion of intra-uterine fluid, wet or dry weights, percentage

water, and absolute or bodyweight-relative values have all been used. Overall, it appears that absolute wet weights generally give acceptable results, although there may be a case for data to be expressed as bodyweight-relative values to overcome potential problems in data interpretation where differences in growth performance occur due to overt toxicity or reduced food intake. Potential problems with the use of simple uterine weight data relate to the observation that there is a biphasic pattern of uterine growth. These phases represent different physiological effects (fluid imbibition versus actual tissue growth). There is also evidence that very high dosages of some xenobiotics may reduce the magnitude of any effects seen (*e.g.* Hisaw, 1959). Indeed, the differences in time course of these different physiological processes are reflected in the schedules adopted, which vary from a single administration followed by recording of uterine weights six hours later (Astwood, 1938) to a dietary administration design in which animals receive treated diet for up to one week (Thigpen *et al.*, 1987a, b, c). Many studies appear to have adopted a three-day treatment period with humane killing on the fourth day of study.

84. Several workers have used other endpoints to monitor uterine growth. These include uterine mitotic rate, histopathological structural changes, uterine DNA and RNA levels, receptor binding, and protein, cyclicGMP or glycogen content. In addition, peroxidase and ornithine decarboxylase activities and oestrogenic and progesterone receptor levels have all been used as alternative endpoints. Sheehan and Branham (1987) suggested that the effects on ornithine decarboxylase activity could represent a separate mechanism to the normal uterotrophic response. In addition, lactoferrin has been identified as an oestrogen inducible protein in the ovariectomised mouse, with elevation of both the protein itself and its mRNA having been quantified following *in vivo* exposure to oestrogens. Indeed, in a review by Teng (1995) this was seen as a sensitive marker possibly suitable for use in detecting environmentally present oestrogenic chemicals and in defining the effects of dose and duration of exposure. This author also pointed out that the lactoferrin model offers the possibility of studying cross-communication between the steroid receptor and the protein kinase pathways. However, this response has only been investigated in a limited number of situations. The role and significance of lactoferrin in the animal is at present unclear, and the influence of other signalling pathways on its production is largely unknown, although epidermal growth factor stimulation has also been shown to elicit production of this protein.

85. The paper of O'Connor *et al.* (1996) is of particular value in assessing the specificity, sensitivity and reliability of several uterine endpoints and their inter-relationships. Although these authors considered that a wide range of endpoints was essential to ensure the identification and characterisation of a chemical's activity and to avoid false positives or negatives, it may be argued that multiplicity of endpoints is likely to result in difficulties in the interpreting the significance of the different positive/negative combinations possible for the various endpoints. In terms of the development of an initial screening method, the use of such a complex test battery would be a questionable use of scarce resources. The majority of the biochemically oriented endpoints reviewed were complex and technically demanding in nature, even in some cases requiring surgery on the animals to implant osmotic pump devices, whilst other endpoints (such as the morphometric analyses) may employ specialised equipment such as image-analysers. Thus, although such a complex test battery is likely to be of great use in the elucidation of the mechanisms of action of a test chemical, the economics of adopting complicated test protocols with multiple endpoints, as a first-level screening tool is not supportable.

86. Overall, there appears to be a strong case for establishing and validating a definitive study design based on the measurement of uterine weight changes in the rat. Efforts should be directed to defining the most sensitive treatment and sampling regimen, using a spectrum of reference chemicals. The question of the preferential use of ovariectomised or immature animals should also be resolved.

Rodent Endometriosis Assay

87. The role of oestrogens in the development and maintenance of endometriosis in women is well recognised. Non-human primate models have been developed but are of course expensive and subject to many problems including ethical and legal constraints and animal availability. A number of workers have successfully established rodent models as suitable for investigating this disease condition. Evidence is also available that such models are capable of responding to a range of chemicals of known endocrine disrupting activity, with either grossly or histologically observable morphological changes or with alterations in hormone receptor profiles.

88. Hening *et al.* (1988) modified a rodent endometriosis model developed by Vernon and Wilson (1985) to permit study of chemical effects on endometrial tissue growth in mature rats. The Hening model involved the initial ablation of a uterine horn with implantation of endometrial sections within the abdomen, followed by subsequent ovariectomy and treatment with the test chemical. After a period of treatment, explants were examined for size and appearance and subsequently histopathology and receptor levels were determined. A similar approach was adopted by Cummings and Metcalf (1995) for mice.

89. Theoretically, these methods could be used as screens for either oestrogenic or anti-oestrogenic activity depending on the study design, because these workers established that the endometrial explants were highly sensitive to oestrogenic stimulation. It would, however, be necessary to define which of the various endpoints was most relevant. In addition, the effect of only a small number of chemicals of known activities have been investigated using these models, so that extension to include a wide range of model (reference) chemicals would be required as part of any validation exercise.

90. Adoption of such a model for routine screening purposes would not be without significant problems, including: the relatively long study duration (several weeks); definition and interpretation of endpoints; the limited historical data; and the need to perform repeated surgery on the animals (which, in the case of the mouse, was seen as technically demanding and prone to loss of animals). These methods are certainly of value in investigating the disease condition, endometriosis, where the ability to use both rats and mice offers a tool to permit study of immunological influences. In addition, the models could on occasion prove useful in assisting in the elucidation of mechanisms of action of chemicals shown to be endocrinologically active by simpler initial screening tests. The complex nature of the test, the long study period and the requirement for repeated surgery does, however, preclude its use as a routine screening model for oestrogenic activity.

Rodent Vaginal Assays

91. The responsiveness of the rodent vagina to oestrogenic stimulation is very well established, having been consistently used as a marker for this activity since the early part of the 20th century. High sensitivity and specificity have been shown in both rats and mice, whilst in most models excellent dose-response relationships have been established. The mouse is smaller and cheaper to maintain, but the rat is generally recognised as the more robust experimental model. In addition, the majority of general and reproductive toxicology data, together with metabolic and pharmacological information on a chemical, will be derived from studies in the rat. The use of that animal can therefore be considered to have clear benefits over use of the mouse.

92. Jones and Edgren (1973) referred to work indicating that rodent uterine weight increase was a generally accepted measure of oestrogenic activity for a chemical. There is some indication that the uterus may even be the more sensitive assay, but it has also been shown to respond to other agents not

characteristically oestrogenic (*e.g.* progesterone and testosterone). In contrast, Jones and Edgren stated that whilst androgens and progestins may produce vaginal mucification, the ovariectomised rat can only initiate keratinisation when exposed to compounds normally considered oestrogenic. Thus there is evidence that the rodent vagina may be the more specific model for oestrogenic modulation. Many of the rodent vaginal models reviewed in this document do employ ovariectomised animals. However, a number of workers have shown that immature non-ovariectomised animals are also highly responsive to oestrogenic stimulation. Indeed, Martin and Claringbold (1958) refer to the possibility that immature mice may be more sensitive than ovariectomised adults. Therefore, as for the rodent uterotrophic assay above, a question remains as to whether ovariectomised or immature animals are the most appropriate model.

93. Similarly to the situation for the uterine assay, there appears to be no agreed treatment schedule for this model. Regimens vary from the Allen and Doisy model, involving a total of three injections at four to six hour intervals, to a 14-day treatment period as used by Jones and Edgren. The majority of methods use daily dosing for five days or less. Clarification of the optimal treatment regimen would clearly be required before a method could be accepted for regulatory purposes. Use of groups given a test chemical alone, or in combination with a known oestrogen, permits the investigation of possible agonistic and antagonistic activity.

94. As might be expected, given the long history of work on the rodent vagina, many endpoints have been used to monitor the vaginal response. These include: time of vaginal opening in immature animals; vaginal weight change; the induction of oestrus as assessed by vaginal smearing; washing or histopathological examination; and the use of a number of biochemical markers. From the available information, it appears that vaginal weight may be less sensitive than uterine weight change and the other vaginal endpoints studied. Also, most of the biochemical endpoints used involved complex and technically demanding techniques, although Martin (1960) reported the mouse triphenyltetrazolium assay as simple to perform. This latter method has been little used, with only a restricted range of chemicals investigated, and it would therefore require rigorous validation before adoption. Overall, given the high sensitivity of the established morphological endpoints, it is considered unlikely that there would be any appreciable benefit from adopting a biochemical endpoint for routine screening use.

95. Selection of the most appropriate morphological endpoint is open to debate. Although several methods involving histological processing and light microscopy have been documented, and Martin and Claringbold (1958) stated that their histopathological assessment model was considerably more sensitive and precise than the standard smear assessment, these histopathological procedures are clearly more demanding of resources and technical skill. In addition, vaginal smear assessment is rapid, allows repeated sampling or use of the same animal, and has been found to provide reliable, sensitive data under normal laboratory conditions. Indeed, the use of vaginal washes, with slide preparation and examination, was included as the sole vaginal endpoint in the test battery proposed by O'Connor *et al.* (1996). This technique may therefore be preferable as a first-level screening tool. The ease of measurement and sensitivity to oestrogens of vaginal smearing also suggests that consideration should be given to the wider inclusion of vaginal smearing/washing in routine short-term toxicity studies.

Rodent Mammary Gland

96. In a model developed by Brown and Lamartinière (1995), the oestrogenic activity of a small number of previously highly characterised chemicals (DES, genistein, Arochlor 1221 and 1254, *o,p'*-DDT and TCDD) was investigated in the female rodent mammary gland following daily subcutaneous injection of the animals for four days. Several endpoints were used, including: size of the mammary gland; terminal

ductal structure; and proliferative and labelling index. In the protocol used it was apparent that statistical analysis was relatively unsophisticated and that the group size was small.

97. Quantifiable effects of the known oestrogenic and anti-oestrogenic chemicals were shown, although difficulties in selecting of the dosages and treatment regimens were reported by the authors. It must also be stressed that this test does not provide detailed mechanistic information on the mode of action of a chemical. The method as reported does benefit from being an *in vivo* model of short duration, but the endpoints used are relatively specialised. Thus, although the terminal ductular structure was assessed subjectively by histological examination, others (*e.g.* mammary gland size) relied on automated data capture techniques. Although all or part of the methodology contained in this study could conceivably be developed into a short-term screen for oestrogenic or anti-oestrogenic activity, it would first be necessary to validate the robustness of the system using a range of model (reference) chemicals at multiple sites and using personnel from other laboratories. In addition, there is a question as to the cost of the test relative to other, more established models. Given these reservations, this model cannot be recommended for further consideration.

Mouse Prostate Models

98. A number of workers have investigated the effects of fetal or neonatal exposure to sex hormones and synthetic oestrogens on the development of the mouse prostate (Pylkkänen *et al.*, 1991, 1993; Mäkelä *et al.*, 1995a, b.; vom Saal *et al.*, 1997). The basis for the model is the belief that oestrogenic exposure at this early stage of development can lead to permanent inhibition of the androgen-dependent growth and function of the prostate. This model therefore offers the possibility to study the effects of chemical exposure at a critical developmental stage. In the basic model, the neoDES mouse is prepared by subcutaneous injection of diethylstilboestrol (DES) for varying periods over the first nine days of life. Alternatively, the fetuses may be exposed by treatment of the dam with oestradiol or diethylstilboestrol during gestation. At varying times thereafter, animals are killed and the prostate (and, on occasion, other accessory sex organs) examined. In refinements to the basic model, the effect of castration of the animal at two months of age and subsequent treatment with oestrogen or androgen, or the long-term effects of neonatal treatment (> one year), were investigated. Mäkelä *et al.* have also demonstrated the response of adult neoDES mice to phytoestrogens. A wide range of endpoints have been used including: weight recording and histological appearance; oestrogen receptor profiling; thymidine incorporation rate; and *c-myc* expression. vom Saal *et al.* used computer-assisted three-dimensional reconstruction to examine the effect of prenatal oestradiol on fetal prostatic bud formation and also prostate androgen receptors in adult male mice.

99. The work reviewed has shown that neonatal exposure of male mice to oestradiol or DES produces changes in the animal that can be detected throughout subsequent life stages and offers the possibility of an animal model that is sensitive to subsequent exposure to sex hormone-disruptive chemicals including oestrogenic, anti-oestrogenic and, to a lesser extent, anti-androgenic agents. The range of sensitivity and the specificity of response have yet to be systematically investigated. A wide range of study designs have been used to date. Several of the endpoints used have been shown to provide reasonable results (including changes in organ weight, histopathological and androgen receptor level assessment, and gene expression measurement), although requiring varying degrees of technical sophistication.

100. Although these animal models allow the investigation of a chemical's potential to elicit adverse effects during perinatal exposure and have identified a number of potentially sensitive endpoints, a definitive test protocol suitable for use for as a screen has not yet been established and the chemicals

investigated are limited in number and scope. The procedures also appear to be relatively demanding and the study duration protracted. Thus, although the models are likely to be of value as a research tool to investigate the developmental processes occurring during the perinatal period and may be of assistance in clarifying the detailed mechanism of action of specific chemicals, they do not appear to be suitable for use as general screening tools.

Rodent Behavioural Modification

101. Tests for non-specific chemical interference in the mating behaviour of adult rodents have been reported by Hsu (1990) and Menard *et al.* (1992). In these tests the pattern of lordosis shown by adult ovariectomised hamsters or rats placed in an arena with adult males was monitored following treatment with a test chemical. The range of chemicals studied was restricted, comprising oestrogen, testosterone, androst-treiene-dione, tamoxifen, DDT, methoxychlor and DES. vom Saal *et al.* (1995) reported on a method for studying subtle changes in adult behaviour following fetal exposure to oestrogenic chemicals. This involved treating dams during days 11 to 17 of gestation. Male offspring were then maintained for 60 days before being individually housed for a further four weeks. Their urine marking activity was then assessed in a test area. The endpoint for this test was the number of urine (scent) marks produced during a one-hour period. Thus the model was relatively simple, did not require any specialised equipment, and permitted assessment of common behaviour in rodents that has important consequences for the animal's social interaction and reproductive success. The authors only reported on a limited number of chemicals (DDT, DES and methoxychlor) but observed that the test appeared to be highly sensitive, being capable of detecting effects arising from very low exposure levels during the fetal period. It must be stressed that the specificity and mechanistic aspects of this model have not been fully investigated, and that the study period is protracted.

102. Although not sufficiently developed as test systems to be adopted as screening tools, these models serve to underline the complex hormonal interactions involved in the normal social and reproductive behaviour of mammals and the possible influences of chemical exposure. Thus, whilst a chemical may not cause any detectable physical changes in an animal, it may elicit subtle behavioural changes with potentially profound repercussions upon the animal's ability to survive and reproduce. Extrapolation of the significance of such behavioural effects from animals to the human situation is also a significant challenge. Nonetheless, in the light of the potentially profound adverse effects that could be elicited, particularly from low-level *in utero* exposures, there is a need to rigorously examine the behavioural endpoints currently included in reproductive (especially multigenerational) studies and to review the requirements for the assessment of such aspects for different classes of chemical. The marked sensitivity shown to *in utero* exposure strongly suggests that more extensive screening of chemicals using such protocols may be appropriate.

3.2.3 Androgen-Related Tests

Chicken Cockscomb Topical Bioassay

103. In this assay, as reported by Dorfman in 1958, the anti-androgenic activity of a limited number of chemicals was demonstrated by daily dermal administration for six days to testosterone-primed cockerels from day 2 of age. Hennessey *et al.* (1986) modified this protocol, using an extended treatment period of 15 days. The ratio of the weight of the comb to bodyweight constituted the endpoint.

104. This is a simple, rapid *in vivo* model that appears to allow identification of the anti-androgenic activity of a chemical. The authors reported a good dose-response relationship using a single non-

subjective endpoint. An investigation into the sensitivity of the comb to direct dermal application of an androgen would be of interest, because this might permit the assay to be used to monitor for androgenic as well as anti-androgenic activity. Reservations concerning the use of this model include the limited range of chemicals examined to date, the large number of animals used in the current test design, the lack of any clear mechanistic information from the test and, because of the dermal administration route used, questions as to the predictivity and interpretation of data generated due to potentially confounding factors such as absorption, metabolic activation or inactivation, bioaccumulation and excretion.

105. Before such a model could be considered as a short-term screen it would be necessary to standardise the protocol and validate it at multiple sites for a range of model (reference) chemicals. Assessment of the cost of the test relative to other possible systems would also be necessary.

Quail Uropygial Gland Assay

106. In a similar manner to the above model, the uropygial gland of the castrated male quail has been shown to be sensitive to anti-androgenic chemicals (Daniel *et. al.*, 1986). The effect on androgen receptor levels in the gland and dodecanediol in the secretory products are compared for animals treated with the test material and with testosterone, either separately or in combination. Both single and multiple administration protocols were reported.

107. This model, even when only a single administration is given, is reported to show anti-androgenic activity and to establish dose-response relationships. It is also recognised that it may be possible to modify the protocol to investigate androgenic as well as anti-androgenic effects of chemicals. A potential drawback to the use of this model is that it only addresses the local activity of a compound on the uropygial gland and therefore does not address systemic aspects such as absorption, metabolic activation or inactivation bioaccumulation and excretion. The range of chemicals investigated is currently also very restricted. Given these limitations, this model cannot be recommended as a screening test at this time.

Rodent Submandibular Gland Sensitivity

108. The effect of androgen exposure on the development of the murine submandibular gland was investigated by Katsukawa *et. al.* (1989). In these investigative studies, young intact or gonadectomised mice were treated for five days with a range of androgens. The effect on submandibular esteropeptidase, 5 α -reductase and α -hydroxysteroid dehydrogenase activities or nuclear binding of 5 α -dihydrotestosterone was assessed.

109. This model was not developed with the intention of being used as a screening tool, but it does show that sex hormones may be involved in the functioning and development of tissues not usually associated with their principal role. Thus it is possible that sex hormone-disruptive chemicals could affect a wide range of unexpected organs and endpoints within an organism, and this underlines the need to include *in vivo* tests in any assessment strategy. Clearly, reliance solely on highly focused *in vitro* models would not provide adequate coverage of all the possible interactions of a chemical within an organism and would thus prevent a satisfactory assessment of the hazard posed by that chemical.

Rat Prostate Models

110. In addition to the highly specialised mouse models discussed above, developed to assess the role of oestrogen and oestrogenic substances on prostate growth and development, many workers have

developed alternative rodent prostate models based on the rat to assess the androgenic or antiandrogenic activity of a chemical. A wide range of treatment regimens and endpoints of varying complexity have been reported.

111. Many workers have simply used prostatic weight as an endpoint of androgenic potential. Many chemicals have been used in such studies, and both intact (Chen *et al.*, 1988) and castrated (Phillips *et al.*, 1990, and Kuhnz and Beier, 1994) rats have been used. Treatment periods of between seven and 13 days were reported as satisfactory. By comparison of the response to a chemical when given alone or in combination with testosterone, it proved possible to show both agonistic and antagonistic activities. In some cases, additional endpoints such as seminal vesicle weight and DNA/RNA content were included in the study design. These studies have shown that androgenic and anti-androgenic effects of chemicals may be rapidly and reliably established by comparing the weights of the ventral prostate or seminal vesicles of treated and untreated animals. In these studies the use of castrated animals permits greater control of the model system and facilitates detection of androgenic activity. It must, however, be recognised that weight increases in these organs may occur as a result of other mechanisms, *e.g.* tissue oedema or inflammation. This would probably not be a significant problem if such a test were adopted as an initial screen, but where it is necessary to firmly establish the trophic nature of the response and to provide a rigorous quantitation of growth, the use of DNA and/or RNA measurement could prove valuable. It should also be remembered that recording the weights of the accessory sex organs in regulatory rodent toxicology studies may also act as an initial alert to possible sex hormone homeostatic disruption.

112. One point to be considered relating to the use of accessory sex organ weights to assess androgenic activity is that age at exposure may significantly affect the weight response seen. This possibility was demonstrated by Cook *et al.* (1993) as part of an investigation into the mechanisms of Leydig cell tumourogenesis by linuron (Section 0). Treatment of rats with either linuron or flutamide was found to result in different organ weight and serum sex hormone level responses, depending on whether immature (days 32-45) or mature (days 93-107) animals were considered. The authors did not investigate whether the differential effects apparent in the older animals were unique because they were first treated at this age, or would have developed anyway with age in animals first treated whilst they were young. Potentially, if such differential responses with age of first exposure were found to be widespread and significant in degree, there would be a need to consider the impact of such varied susceptibilities on the approaches adopted in the various testing programmes.

113. Other more complex endpoints of prostate function have been used in specific studies. Botella *et al.* (1987) investigated the anti-androgenic potential of a number of progestins when given by intraperitoneal injection to groups of five castrated rats. The prostates were removed from the animals one hour after treatment, and cytosolic and nuclear androgenic receptor binding levels in the prostate were assessed using radioassay techniques. Chemicals investigated included mibolerone, medroxyprogesterone, nomegestrol, megestrol, chlormadinone and cyproterone. This model permitted a mechanistic investigation of a chemical's interference with the androgenic hormonal system following *in vivo* administration. The system was clearly restricted to a single mode of action. It required specialised equipment and a degree of technical competence. In addition, it has only been used on a limited number of chemicals displaying known anti-androgenic activity. As a result, this system does not appear to be useful as a routine screen for sex hormone-disruptive capacity. Work by Fjösne *et al.* (1992) suggested that it might be possible to use the change in ornithine decarboxylase activity in the ventral prostate of castrated rats as a highly sensitive marker of androgenic disturbance. Effects were apparent 24 hours after a single administration of testosterone. Considerable work would, however, be required to validate this technique as a definitive and reliable marker of androgenic disturbance and to assess its relative sensitivity and specificity compared to other *in vivo* and *in vitro* models.

Mouse Seminal Vesicles

114. Broulik and Horky (1988) used seminal vesicle weight changes in the castrated mouse as a marker for the androgenic activity of an anti-hypertensive vasodilator, minoxidil, which had been seen clinically to cause changes suggestive of androgenicity. After 21 days of treatment, the seminal vesicle weight was shown to be highly responsive to the presence of androgenic agents. Information was not presented as to the sensitivity of this model to varying dosages, and the potential for interactions with other hormonal disrupting agents (such as anti-androgens) was not investigated. The study was protracted compared to those shown to be effective for monitoring prostate weight change in the rat, but this model again shows the responsiveness of accessory sex organs to sex hormone-disruptive chemicals. It is nonetheless considered that a rat will generally prove more suitable for screening than a mouse, given that most of the other pharmacology, toxicity and metabolic data generated for a compound is likely to relate to the rat.

Hamster Flank Organ

115. Brook *et al.* (1991) and Cabeza *et al.* (1995) reported on the use of the flank organ of the male Golden Syrian hamster as a marker for anti-androgenicity for a total of four chemicals. Castrated animals received maintenance injections of testosterone and the test chemicals were dermally applied to one of the flank organs, the other remaining untreated as a within-animal control. Treatment durations of seven to 21 days were used, so these are relatively long studies. Measurement of the size of the flank organ was shown to allow quantification of topical androgenicity or anti-androgenicity using a simple non-subjective endpoint. It was also been shown to correlate with a number of microscopic and biochemical (glucose and lipid content) changes. The absence of minimal response reported in the contralateral flank organ and prostate indicates the absence of significant systemic absorption in the chemicals studied to date. Thus it is likely that this model would not detect a chemical requiring metabolic activation. Bioaccumulation, metabolic inactivation and excretion rates are other significant factors not addressed. At present the range of chemicals investigated using this method is very restricted and its sensitivity and specificity are unclear. In addition, reproducibility across a range of laboratories is unknown. On the basis of these reservations, it is not possible to recommend this model for adoption.

Dog Prostate Ultrasonography Model

116. In a novel approach to monitoring prostatic responses to anti-androgenic treatment of the dog, Cartee *et al.* (1990) reported on the use of an ultrasound scanner to image the size of the organ. Calculated cross-sectional areas were obtained from scanned images at intervals during a 47-day treatment period, using flutamide or hydroxyflutamide in an attempt to monitor prostatic involution. In addition to the obvious implausibility of adopting a dog-based model as a screening test, it appears that there are several problems associated with the actual measurement technique: specialised equipment and trained staff are required; considerable day-to-day within-animal variation in prostate size was seen; and a between-group statistical difference from the controls was only established for the highest treatment level used in this study. In contrast, routine histopathology or measurement of testicular size showed changes at each treatment level. Although it is interesting to speculate as to whether future technological developments in ultrasound scanning technology would improve performance, the current information shows that the in-life use of ultrasound monitoring is of insufficient sensitivity to be of value except perhaps in very specialised circumstances where the time course of prostatic size changes has to be established.

Dog Sialic Acid Depletion Test

117. Jain and Dixit (1986) established that sialic acid concentration in the gonads, epididymides and prostate of dogs was highly dependent on hormonal stimulation being influenced by both testosterone and a phytoestrogen. Data are not available on the sensitivity of this model to varying dosages and the reasons for selection of a 60-day treatment period. In any case, its use is precluded on the basis of the species used. In addition, the endpoint involved destructive sampling of the animals, precluding time course measurements within an individual. There was a reference in the paper indicating that a similar dependency to sex hormones may exist in the rat, although information on sensitivity and specificity was not provided.

3.3 RELEVANT *IN VITRO* METHODS FOR POTENTIAL EFFECTS IN HUMANS**3.3.1 Non-Specific Test Methods*****Human cell line screens***

118. A number of human-derived, established cell lines have been used to study a wide range of hormonal activities. Those relating to oestrogenic activity are reviewed in Section 0 below. Some models use the cell lines in unmodified form, whilst others modify their sensitivities or responses by the use of either stable or transient transfection techniques. There are severe limitations implicit in all currently available *in vitro* cell assay systems. These limitations, which apply to all models, will be discussed later.

- **T-47D cell lines**

119. A microplate titre system based on progestagen induction of alkaline phosphatase activity in the T-47D cell line has been developed by Markiewicz and Gurpide (1994). They reported the results of tests on a number of synthetic and naturally occurring chemicals, including progesterone and diethylstilboestrol. The test involved the incubation of cells with the test chemical for 72 hours, followed by assay for alkaline phosphatase activity using *p*-nitrophenolphosphate. The authors reported being able to distinguish between full and partial agonists with this system. A further development using this cell line was reported by White *et al.* (1994a), in which both progestational and androgenic activities were measured for a number of progestagens, dexamethasone and dihydrotestosterone. This was achieved by the stable transfection of a DNA cassette containing a synthetic steroid-inducible promoter in an Epstein-Barr episomal vector with expression by a bacterial chloramphenicol acetylase (CAT) gene. Activity was determined using thin layer chromatographic separation or *in situ* colourimetric determination. The low background activity and high inducibility by progestagens suggested that a pGRE5-CAT/EBV vector-transfected T47-D cell line could be of value for investigating steroidal compounds. Advantages of such vectors include a high frequency of clone isolation and absence of expression modulation as a result of random integration into chromosomes, because the transgene is propagated on an autonomously replicating plasmid. The demonstration that transcription is strictly dependent on the levels of progesterone or dihydrotestosterone indicated that it was possible to strictly control the expression of this promoter. Based on the limited data available, it is possible that the microtitre plate CAT system might be suitable for screening of potential progestagens and antagonists. Response to progesterone down to 30 pM was recorded, with maximal activation at 100 nM, a range sufficiently broad to enable EC₅₀ determination. A drawback was the low absorbency values obtained with androgen-treated cells, which indicated that that this system was unlikely to be of value for androgenic screening. Although androgenic activity was detectable using thin layer chromatography, this represents a much more time-consuming and demanding process. Given its limited use to date, adoption of this cell line cannot be recommended at present.

- **ZR-75-1 human breast cancer cell line**

120. The cell line ZR-75-1, possessing functional oestrogen, androgen, glucocorticoid and progesterone receptors, was used by Poulin *et al.* (1990) to investigate the degree of progestin-induced inhibition mediated by the different receptor types for six synthetic progestins. This involved complex relative binding affinity studies using a number of radiolabelled chemicals. The assay period was protracted, and the multifactorial nature of this assessment necessitated the interpretation of complex data. Thus, although the authors reported the assay to be of great value in elucidating specific mechanisms of action of the “progestins”, the model does not appear to be suitable as a general screen for sex hormone-disruption. In particular, the possibility that a novel chemical might present with multiple receptor activities would result in great difficulty in interpreting data.

Animal Tissues and Primary Cell Screens

- **Bovine uterine cells**

121. Tiemann and Tuchscherer (1995) reported on the separate culturing of different uterine cell types from cattle and showed them to be responsive to exposure to a small number of organochlorines. The significance of the endpoint measured (DNA synthesis), and its relationship in this model to sex hormone receptor interactions, were not established by the authors. At present neither the method nor its mechanistic basis are sufficiently developed to warrant further consideration.

- **Monkey ovarian model**

122. Bengtsson and Mattison (1989) reported on a culture system using ovarian cells of the rhesus monkey. The cells were shown to be metabolically active following pre-humane-killing stimulation with gonadotrophin. However, the endpoint used was not directly applicable to sex hormone-disrupter activity. Given the species used and the nature of the model, it is not considered suitable for further consideration.

- **Rodent ovarian models**

123. Several workers have reported on the use of *in vitro* ovarian tissue culture models, including techniques to produce cultures rich in specific cell types. These studies were basic scientific investigations rather than attempts to establish screening models. Examples of study aims include the study of effects on cellular metabolic and functional capacity of oestradiol, lutenising hormone, forskolin, cholera toxin, vasoactive intestinal peptide and isobutylmethylxanthine and clarification of the bioactivation of dimethylbenz(a)anthracene. As might be expected, a wide range of endpoints were used.

124. It is clearly feasible to establish *in vitro* cultures of primary ovarian cells of rodent origin for relatively short durations, although in some cases procedures were relatively involved and demanding. Evidence of biochemical responsiveness was demonstrated in some rat models which presents the interesting possibility of studying the role of biotransformation within the gonad. The method of Bengtsson *et al.* (1992) for culture of enriched populations of cells of a particular type would be of particular interest in this respect.

125. Overall, these models may prove of value for detailed mechanistic studies but are not suitable for chemical screening.

- **Animal Cell Line Screen**

126. A mouse hepatoma-derived cell line, HepalC1c7, which endogenously expressed the arylhydrocarbon (Ah)-receptor, was used by Zacharewski *et al.* (1995) to assess the Ah-mediated, dioxin-like, activity of pulp mill effluents. In this assay, cells were transfected with a luciferase, dioxin-responsive element regulated-reporter. Cells were given 24-hour chemical exposures before analysis for luciferase activity; results were reported relative to the response from a standard dioxin concentration. The AhR bioassay was shown to be able to detect dioxin-like activity in highly complex mixtures, with a detection limit equivalent to 80 pg TCDD per plate. This assay may be of interest for the screening of complex industrial effluents for Ah-activity but, as in the case of most *in vitro* assays, it can only identify chemicals capable of directly interacting with a particular receptor type and does not address other potential effects. The potential application of this system is therefore considered to lie in specific rather than general application.

3.3.2 Oestrogen-related test systems

Human Primary Cell Screens

127. Casslén and Harper (1991) developed a culture technique to grow primary human endometrial cells taken from uteri removed because of benign disorders, and demonstrated a dose-related response to oestradiol by measurement of PDG_{2α} levels. Using this technique, cells retained normal morphology when maintained in serum-free medium for up to five days. Another method, developed by Markiewicz *et al.* (1993), showed altered PDG_{2α} levels in cultured endometrial cells exposed for 24 hours to combinations of oestradiol, equol and hydroxytamoxifen. Both these models appear able to respond to hormones posing oestrogenic or anti-oestrogenic activity and may be a useful tool for detailed study of human primary cell function. The culture procedure of Markiewicz *et al.* was notably less involved and of shorter duration.

128. A paper by Kruk *et al.* (1990) demonstrated that it was possible to develop a sampling, storage and culture technique that would permit culture of human ovarian epithelium tissue, although the sensitivity of the cells to hormonal stimulation was not investigated.

129. The inference from these studies is that it might theoretically be possible to develop an *in vitro* test battery based upon a number of human tissue types to screen for chemical modulation of cell function. Although such a model might initially appear of great relevance to human safety evaluations, several significant problems exist: the similarity of the cellular metabolism and physiology to that found *in vivo* is unknown; such tests do not address the cyclical nature of changes within the reproductive organs; the responsiveness of cultured cells to a wide range of chemical types is unknown (in these studies highly active hormonal substances were used); and the applicability of the endpoint to processes within an intact organism is unclear. Additional questions relating to availability, safety and consistency of responses are also outstanding. Such human primary cell systems are clearly unsuitable as a short-term screen, but they may play a role in mechanistic studies on cell functions.

Human cell line screens

- **MCF-7 cell line systems**

130. The MCF-7 cell line has been the subject of considerable scientific research into its ability to detect either oestrogenic agonistic or antagonistic activity. A very large number of natural and synthetic chemicals have been tested using this strain, including natural and synthetic hormones, some pesticides, PCBs, plasticisers, phytoestrogens and animal feedstuffs.

131. Various workers have developed a range of endpoints to assess the cellular responses of MCF-7 cells, including measurement of the induction of cell proliferation, levels of the progesterone receptor or its mRNA, specific exoproteins (*e.g.* pS2) or even, in a transfected sub-strain of MCF-7, activity of a luciferase enzyme. It has been reported that the growth response of MCF-7 cells is approximately 10 times more sensitive to oestrogens than the synthesis of progesterone receptors (Welshons *et al.*, 1990) and that the most sensitive index of MCF-7 cell proliferation is incorporation of [³H]thymidine (Desauliniers *et al.*, 1995), although proliferation is more commonly assessed by counting cell nuclei in a Coulter counter. The endpoints used to assess oestrogenic activity in the MCF-7 assay are all oestrogen-dependent, receptor-mediated processes. Chemicals exerting oestrogenic activity through mechanisms other than via interaction with the oestrogen receptor would not be expected to be detected by this model. However, one group has shown that dioxin (TCDD) and a number of PAHs (chemicals which interact with the Ah-receptor) can act as anti-oestrogens in MCF-7 cells by inhibiting oestradiol-induced progesterone receptor binding. It is possible that this is due to cross-talk between the Ah and oestrogen receptor signal transduction pathways (Harper *et al.*, 1994).

132. Human and fetal bovine sera contain an inhibitor of proliferation of oestradiol-sensitive MCF-7 cells. Oestradiol promotes cell proliferation by neutralising this inhibitor (Soto and Sonnenschein, 1985). In order to assess the oestrogenic activity of chemicals in this system, it is therefore necessary to ensure that the medium contains serum stripped of endogenous oestrogens using dextran-coated charcoal. Although such treatment may remove more than 99% of the endogenous oestrogens, a sufficient amount may remain to elicit an oestrogen-dependent response (Zacharewski *et al.*, 1995). Furthermore, some laboratories using oestrogens in cell culture have found that these can contribute to the ambient level of contamination by oestrogens in this assay. This is normally associated with a diminution or loss of responsiveness in the cells, because they are already stimulated to some extent by the “control” medium. One group has suggested that use of anti-oestrogens such as tamoxifen in this assay permits testing of the baseline growth rate of control cells (Welshons *et al.*, 1990). Clearly these potential problems need to be controlled for when using this assay, and standard conditions and test criteria must be specified to ensure that both intra- and inter-laboratory variations are kept to a minimum. A possible drawback to the use of this assay as a rapid screening tool is that the induction of proliferation takes several days to assess. vom Saal *et al.* (1995) used binding affinity to the oestrogen receptor in MCF-7 cells as the endpoint for oestrogenic activity, which takes only 18 hours to perform. In addition, both serum-free medium and 100% serum were used to determine whether the presence of serum proteins alters the relative binding affinity of the test chemical, which may have important implications for effects *in vivo* and is not possible to investigate in longer-term cell cultures. However, this type of assay only assesses binding to the oestrogen receptor and does not discriminate between agonist and antagonist activity following binding.

133. Several authors have suggested that MCF-7 cells could be used for screening chemicals suspected of being oestrogenic (Soto *et al.*, 1991; Mayr *et al.*, 1992) and that they could also aid in the prediction of chemicals’ oestrogenic effects on human health, because target cells and serum are of human origin, the assay is very sensitive (able to detect 3×10^{-11} M oestradiol or equivalent activity), it is easy to perform, and it can be used to screen many compounds over a range of concentrations (Soto *et al.*, 1992).

134. Comparing *in vitro* MCF-7 data with results from *in vivo* test systems, Mayr *et al.* (1992) found a similar order of relative potencies comparing exoprotein induction in MCF-7 cells with the uterotrophic

assay in mice when testing various mycoestrogens and phytoestrogens. However, the *in vitro* assay tended to indicate higher activities, the lower activities *in vivo* most likely being due to compound metabolism. Indeed, the metabolic capacity of MCF-7 cells is not well understood. Although many chemicals have been reported to affect oestrogen-dependent endpoints, it is often not clear whether the active oestrogenic agent is the parent compound itself or one or more metabolites. PCBs, which may require metabolic activation to elicit oestrogenic activity, are not active in this system (Soto *et al.*, 1992). Thus to identify chemicals requiring prior metabolism before exerting oestrogenic activity, it would be necessary to include an incubation step with liver microsomes or some alternative metabolic activation system either before or in combination with the assay. Soto *et al.* (1995) stated that, using MCF-7 cell proliferation assay (or the E-screen), no false positives or negatives were observed among the oestrogens and non-oestrogens tested. However, it is not clear what test(s) was/were used to define oestrogenicity for this comparison as it was also stated by these authors that, for animal bioassays, there is no “gold standard” for oestrogen action. Other authors have found the endpoint of cell proliferation to be responsive to several non-oestrogenic mitogens including bile acids, insulin-like growth factors, and epidermal growth factor. As a result of these observations, it has been proposed that the assay suggests, but does not equivocally demonstrate, the oestrogenic activity of a substance (Zacharewski, 1997). MCF-7 cell proliferation has also been shown to be sensitive to caffeine and ethanol (Jones *et al.*, 1997a). In addition to differences in metabolism, it has been suggested that some discrepancies between the potency of chemicals in the MCF-7 assay and animal assays *in vivo* are due to the persistence and bioaccumulation of chemicals in animals (Soto *et al.*, 1995), which clearly cannot be considered in *in vitro* assays of this type.

135. A further development of the standard MCF-7 assay has been the use of either stable or, more commonly, transient transfections of these cells with recombinant oestrogen receptor/reporter genes. Pons *et al.* (1990) developed a stably transfected MCF-7 cell line which measures oestrogen-dependent induction of luciferase activity as bioluminescence. The authors used this system to detect oestrogenic and anti-oestrogenic activities. Using apparently similar cells, von Angerer *et al.* (1994) were unable to detect weak oestrogens (agonists) because of a high level of basal luciferase activity. In contrast, Pons *et al.* found the system to be highly sensitive and suggested the assay could be used as an early screening test for new oestrogens or anti-oestrogens for a number of reasons:

- a) Luciferase was expressed in response to oestradiol exposure in a dose-dependent manner with an EC_{50} lower than 10^{-10} M.
- b) Oestrogen induction of luciferase was not shown in oestrogen receptor negative cells transfected with the plasmid.
- c) The measurement is quick (on sonication, luciferase activity lasts about 15 seconds per tube).
- d) The assay is highly sensitive, with between 10^5 and 10^6 cells needed for a measurable inducible activity.

136. Using this system, oestrogenic or anti-oestrogenic activity can be measured only 24 hours after incubation of cells with test compound. No cross-reactivity was demonstrated with ligands of the “superfamily” receptors.

137. Zacharewski *et al.* (1995) used MCF-7 cells transfected with the gene coding for the oestradiol-inducible protein, pS2, linked to a luciferase reporter, and examined the ability of various complex mixtures to antagonise oestradiol-induced luciferase activity. The oestradiol response element in the pS2

gene has a dioxin response element located upstream, enabling the detection of chemicals capable of eliciting an Ah receptor-mediated anti-oestrogenic response.

138. Thus it might appear that the transfected MCF-7 cell bioassay addresses some of the limitations of other *in vitro* assays, in that it has been reported to be sensitive, discriminating and amenable to large sample numbers. It has been suggested that this type of bioassay can be used in the assessment of complex mixtures by (1) complementing chemical analysis (indicating the presence of compounds that may not be detected by GC/MS because of the lack of appropriate standards) and (2) providing an indication of the toxic potency of a sample. Furthermore, these assays could be used as a screen for identifying active ligands following fractionation of a complex mixture, and for comparing potencies of environmental oestrogens and dioxin-like compounds. Nonetheless, concern has recently been expressed about the use of this cell line because of problems with the reproducibility of data derived from the multiple sub-clones that exist. For example, Villalobos *et al.* (1995) conducted a study using four different, but widely used, clones of MCF-7 in which the cells were cultured in identical media. Experiments were run in parallel, although it proved impracticable to use cells of exactly the same passage number. The results obtained showed distinct differences in the proliferative response to oestradiol and to the xenoestrogens, p-nonyl-phenol and bisphenol A, by the four cell stocks. MCF-7 BUS displayed the greatest sensitivity, whereas MCF-7 BB104, MCF-7 BB and, in particular, MCF-7.ATCC displayed poor sensitivity and dose-response relationships. Differences in response could not be attributed to variations in culture conditions or passage number. In contrast, Jones *et al.* (1997b) reported a large variation in the sensitivity of the proliferation assay using different assay conditions, with the maximum proliferation induced by 17β -oestradiol varying from 200 to 400% or more of control levels. Given this variability in response, the authors urged caution in the interpretation of such assays and emphasised the need to identify selected cell strains and to standardise protocols. Thus, for an assay to be effectively validated, a standard set of parameters that measure reproducibility must be developed. In evaluations of screens using cell lines (*e.g.* the E-screen), cell stock uniformity and culture conditions are the most important variables affecting reproducibility.

139. Despite these significant problems with MCF-7 cells, the volume of data available for these cells indicates that they should be included in further development programmes, even if only to provide a baseline comparator. In addition, it must be recognised that although these problems have been highlighted in respect of MCF-7 cells, similar problems could potentially exist in any established cell line.

- **Ishikawa cell line systems**

140. The paper by Holinka *et al.* (1989) demonstrated that exposure to oestradiol would elicit effects in the Ishikawa cell line even in the absence of serum in the medium; endpoints included cell colony formation, autoradiography using thymidine-labelling, and metaphase analysis. Of the parameters measured in this model system, cellular proliferation over the exponential growth phase appeared to be the least demanding technically and provided data suitable for statistical analysis. Effects on colony formation were found to be dependent on the co-incubation of serum factors, indicating that this is unlikely to represent a direct effect and may be more susceptible to culture conditions. The assessment of mitogenic effects involved the use of radiolabelled material and calculations that required either further studies or acceptance of certain assumptions relating to stages in the cell cycle. The work of Ignar-Trowbridge *et al.* (1993) suggests that endogenous chemicals, such as peptide growth factor, that are not oestrogens are also capable of stimulating the oestrogen receptor sites. However, the significance of this finding for the screening of xenobiotics using oestrogen receptor containing cell lines is questionable.

141. The papers of Littlefield *et al.* (1990) and Markiewicz *et al.* (1993) reported on the use of an

assay system for this cell line in which alkaline phosphatase activity (visualised by measurement of a *p*-nitrophenol colour response) was the endpoint for oestrogenic activity. Chemicals investigated included natural and synthetic oestrogens, androgens, glucocorticoids, mineralocorticoids and the $\Delta 5$ - 3β -hydroxy steroids, biochanin A, hydroxytamoxifen, and a number of phyoestrogens. This work indicates that the system may represent an easy, rapid *in vitro* assay. Evidence was presented that this model was capable of demonstrating dose responsiveness and relative potencies similar to those of *in vivo* systems, and that it could also be used as a detection system for anti-oestrogenic activity. A wide range of oestrogenic chemicals of steroidal and non-steroidal structure have been tested, and the findings indicate that other classes of steroid hormone do not elicit any significant cross-reactivity.

142. In order to firmly establish the suitability of this test system, it would be necessary to validate this model against a wide set of model chemicals in a range of laboratories and to compare the sensitivity and specificity against any other appropriate *in vitro* model. In addition, given the problems identified for the MCF-7 cell line, it would be necessary to establish whether sub-clones exist for this cell line and whether the reproducibility and responsiveness vary between cultures at different laboratories or over time.

- **MDA-MB-231 CL10A cancer cell line**

143. In a study by Jaing *et al.* (1993), the use of stable transfectants of an oestrogen receptor (ER)-negative breast cancer line, MDA-MB-231 CL10A, facilitated a structure-function analysis of the ER. After an eight-day exposure period, total DNA concentration of the cell cultures was measured as a marker for proliferation and growth. By comparing the structure of anti-oestrogens and their relative activities in wild and mutant ER transfectants, it proved possible to predict the activity of some anti-oestrogenic compounds. Of particular note was the inhibitory nature of the responses to oestradiol, which may be peculiar to this cell line. The proliferative endpoint of the assay was considered advantageous, because it represented a physiological endpoint. The method may, however, be criticised for the relatively lengthy incubation period required and the complexity of the assay. de Cupis *et al.* (1995) also reported on a comparison of four other lines and MDA-MB-321 cells using two pure non-steroidal antioestrogens and the antioestrogen, tamoxifen. Several endpoints were investigated which confirmed that this basic form of the cell line was unresponsive to oestrogenic stimulation. Given the limited data available, it is not yet possible to characterise the robustness, sensitivity or specificity of the various sub-forms of this cell type. It will therefore be excluded from further consideration.

- **T-47D cell lines**

144. Welshons *et al.* (1987) have investigated oestrogen agonistic and antagonistic activities of enterolactone, enterodiol and equol using the human breast cancer cell line, T47D. Six-day exposure periods were used, with subsequent measurement of progesterone receptor levels and DNA level. Watanabe *et al.* (1990) included this cell line in a MINI assay in which the effects of anti-oestrogens, given separately or in combination with oestradiol, were studied using a range of endpoints including thymidine incorporation, ER and progesterone receptor levels, and DNA content. Assay systems for oestrogenic agonistic or antagonistic activities utilising this cell line appear to show good sensitivity in the models reviewed, although the inhibition from non-specific toxicity recorded at high concentrations together with the low responsiveness seen in the MINI method of Watanabe *et al.* may present limitations. It was suggested that the potential problem of false negatives was soluble by inclusion of an oestrogen rescue process which, if unsuccessful, would indicate the presence of a toxic response that was not oestrogen-mediated. These studies underline the dependency of an assay's sensitivity on the frequency of ER expressed by a particular cell line. This is a major concern for all receptor-mediated cell assays. A further limitation of the MINI assay, as highlighted by Watanabe *et al.*, is the failure to correlate *in vitro* results using cultured cells taken from patients with the clinical responsiveness to treatment with anti-oestrogenic compounds subsequently shown by the patients. This clearly shows, in a human context, the difficulties in extrapolating results from *in vitro* to *in vivo* situations. As with all *in vitro* assays, criticism may be made of the lack of provision for metabolic capacity in the system and the problems in inferring that receptor interaction will result in physiological effect.

- **HeLa cell lines**

145. The HeLa cell line is normally devoid of ER expression, but it has been used with transfected reporter gene constructs to study oestrogen responsiveness. Under these conditions, it has been shown possible to produce cells showing high specificity for chemicals capable of interacting with the ER. Berry *et al.* (1990) investigated differences in the detailed agonistic/antagonistic profiles of two anti-oestrogens using a transiently transfected HeLa cell line with a chloramphenicol acyl transferase (CAT) reporter construct providing the endpoint, whilst Crombie *et al.* (1994) reported on the use of a similar transfected cell to determine the specificity for expression of one isozyme of creatinine kinase (CK) when a range of receptor types were stimulated. Oestrogen-specific CK activity was established, and this was regarded as a suitable marker for oestrogenic activity and for detecting unopposed oestrogen action associated with progesterone antagonism. However, the occurrence of promoter context-dependent activity focuses attention on the highly complex nature of receptor-mediated interactions and indicates that this must be taken into account when interpreting results of any *in vitro* assay. The effect on creatinine kinase has the advantage of being of physiological significance, as opposed to assays which simply detect binding and transcriptional activation. Despite these points, a suitable permanently transfected form has not been characterised. At present, this model is not considered to be developed sufficiently to permit progression.

Summary of assessment of human cell line screens

146. To date, the most commonly used human-derived cell line for the assessment of oestrogenic activity appears to have been the various sub-clones of MCF-7 cells, in either normal or transfected forms. The Ishikawa cell line has also been used for a range of chemicals, although by only a few workers. Nonetheless, this latter cell line does appear to have been developed into a promising assay system using alkaline phosphatase activity as an easily monitorable endpoint. Data exist for several other cell lines, but

they have generally only been used for detailed mechanistic studies on a limited number of chemicals or for receptor function characterisation. Hence their potential application as screening tools remains highly speculative.

147. In both MCF-7 and Ishikawa assays, oestrogen-dependent endpoints have been used to measure the effects and potencies of various synthetic and naturally-occurring chemicals with oestrogenic or anti-oestrogenic activities. Both assays appear highly sensitive. Dose-response relationships have been well established for many chemicals, both natural and synthetic. However, the use of proliferation as an endpoint in MCF-7 cells has been shown to be responsive to non-oestrogenic mitogens and therefore may only suggest, rather than demonstrate, oestrogenic activity. In addition, in common with all *in vitro* assays for oestrogenic activity, these tests suffer from a number of limitations in terms of their application as a screening method, not least of which is the time required to perform the tests (several days). In this respect, the transfected MCF-7 cell assays, which measure luciferase activity as the oestrogenic endpoint, may warrant further investigation because they permit a rapid assessment of potential activity. Against their adoption, however, is their complex nature and the requirement for relatively sophisticated equipment. At this stage, both MCF-7 and Ishikawa cell lines are considered to warrant further investigation, development and evaluation.

Animal tissues and primary cell screens

- **Rat uterine cell culture**

148. *In vitro* cultures of dispersed rat primary uterine cells were used by Kassis *et al.* (1984) to study responses to oestradiol, progesterone, testosterone and dexamethasone. Following the cells' establishment in culture plates, they were exposed to the test chemical for up to six days, followed by assay of the cells for progesterone and oestrogen receptor expression and protein. In a somewhat different approach, Galand *et al.* (1987) exposed uterine horns taken from rats to oestradiol or to two DDT congeners for two hours *in vitro*, followed by measurement of an inducible 130K protein and of cGMP levels.

149. Both methods reviewed were part of detailed mechanistic investigations rather than being intended to establish a screening model. Each involved relatively demanding biochemical processing of samples and have only been used for a limited number of chemicals. The method of Kassis appeared to respond specifically to oestrogens, except at very high exposure levels when evidence of cross-talk was seen: for the oestrogen studied, a good dose-response relationship was clearly shown. In the results obtained by Galand *et al.*, however, one of the endpoints investigated in the *in vitro* model did not correlate with other *in vivo* data generated. This again underlines the potential problems in extrapolating between *in vitro* and *in vivo* models. Overall, neither uterine culture model can be considered to warrant consideration as a routine screening test.

- **Rat pituitary cell culture**

150. Lieberman *et al.* (1978) used a culture of rat pituitary cells to compare prolactin synthesis in response to treatment with oestradiol, diethylstilboestrol, progesterone, testosterone, hydroxyandrostanoone or corticosterone. Further work by these authors (Lieberman *et al.*, 1983) using a similar cell culture system investigated the effect of tamoxifen and some of its metabolites alone or in combination with oestradiol, whilst pituitary cell culture was used by Jordan *et al.* (1986) to investigate binding efficiencies

of triphenylbutenes. These pituitary cell models appear to be rapid, reliable *in vitro* culture systems for the detailed mechanistic study of the relative activities of oestrogenic and anti-oestrogenic chemicals that act via oestrogen receptor interaction. They have, however, been used on a relatively restrictive range of chemicals and require technically competent staff working with relatively sophisticated equipment and radiolabelled steroids. They were not developed with the intention of establishing a screening test and are unlikely to be suitable for such use.

- **Mouse Leydig cell culture**

151. Bilinska (1986) used a culture of mouse primary Leydig cells to investigate their *in vitro* responsiveness to oestradiol using a range of enzymatic, morphological and radioimmunoassay techniques. Only two chemicals were investigated in the study, and the cell culture techniques and endpoints were quite complex. Of the endpoints investigated, only suppression of androgen secretion appeared particularly responsive to oestrogen treatment and even this showed variable sensitivity depending upon the age of the culture (control levels rose from the second to the fourth day and fell thereafter). A chemical's effect on Leydig cells could potentially have great significance for the reproductive system of the male. However, this model was intended as a mechanistic tool and does not appear suitable for development into a screen.

Animal cell line screens

- **Transfected chicken embryo fibroblasts**

152. Chicken embryo fibroblast (CEF) cell cultures have been shown to be responsive to chemicals with oestrogen agonistic and antagonistic activities, following the use of transfection techniques to insert human oestrogen receptor and reporter system (CAT) elements. Berry *et al.* (1990) investigated responsiveness to oestrogen and two antioestrogens, whilst White *et al.* (1994b) studied a number of alkylphenolic compounds. Exposure periods of up to 48 hours were used and CAT activity then measured, with normalisation for transfection efficiency using either a galactosidase or luciferase system. Although the CEF model appeared to be able to detect oestrogenic and anti-oestrogenic activity, it has only been used on a restricted range of chemicals by a few workers. This model depends upon transfection techniques to develop a responsive model, and few data are available with which to assess ease of production/maintenance, reproducibility, stability, etc. Also, such systems are only capable of detecting effects arising directly from interactions with the inserted oestrogen receptor. At present, benefits over the use of a human-established cell line have not been shown.

- **Transformed mouse L-cells**

153. Mayr *et al.* (1992) used a genetically transformed clone derived from the mouse Le42-cell to investigate the oestrogenicity of a number of myco- and phyto-oestrogens. A number of plasmids were transfected into the cells to establish the final oestrogen-responsive model. This included a CAT-based reporter construct to enable CAT activity to be assessed as the model's endpoint. In addition to the plant and fungal products tested, tamoxifen, progesterone, testosterone and an oestrogen were also included for comparative purposes. Despite the initial requirement for transfection and a three-week clonal selection period, this system appeared to offer a sensitive and reasonably simple means of determining oestrogenic

potential. It was possible to discern differential activities within the range 10^{-10} to 10^{-5} M. The authors suggested that the method might be further simplified by use of a luciferase or alkaline phosphatase reporter, which would facilitate routine use. Data are, however, limited and there are no comparative data from other workers with which to assess the robustness and reproducibility of the model. Given the limited use of the system to date, it cannot be recommended for adoption as a routine assay.

Yeast screens

154. In recent years, the development of transfection techniques has enabled many workers to develop *in vitro* models in which oestrogen receptor structures derived from humans or animals were expressed by a yeast, *Saccharomyces cerevisiae*. There are clearly many potential benefits relating to the use of a yeast cell culture system. As a result of its longstanding use as a model for mechanistic investigations and in genetic and mutagenic research and regulatory screens, the basic morphology, biochemistry (*e.g.* well characterised oxidative metabolic capacity), physiology and genetic structure and function of this yeast are well established. In addition, the untransformed organism is widely available, can be easily and rapidly cultured, is easily transformed, and has a wide range of suitable plasmids and promoters available. Other benefits include the ease of selecting and maintaining transformed cells by varying the composition of culture media; the rapid growth rate of the cells; the absence of sex hormone receptor mechanisms in the untransformed organism, and the “non-animal”-based nature of this model. In addition, it has been widely established that the oestrogen receptor-expressing, transfected yeast model is capable of high levels of sensitivity (reportedly higher than that achievable by the MCF-7 cell models; Wrenn and Katzenellenbogen, 1993,) can give close correlation with other model systems, and has been shown to demonstrate dose-response relationships for a wide range of chemicals. Chemicals investigated by yeast models include: oestrogen, oestradiol, oestriol, oestrone, diethylstilboestrol, testosterone, dexamethasone, cortisol, aldosterone, progesterone, various plant extracts and plant hormones, representatives of the major groups of surfactants, triazine-derived herbicides, and antioestrogens such as tamoxifen, hydroxytamoxifen and nafoxidine.

155. Most of the models reviewed used a human-derived ER with β -galactosidase activity as the endpoint. This endpoint permits ease of monitoring by a colour-change reaction, with reliable quantitation. In contrast, although having the same endpoint, the method of Khono *et al.* (1994) used a murine receptor whilst Connor *et al.* (1996) used colony growth as an endpoint. Many other differences exist between the various models, including the wide variety of strains and constructs that have been used and the significant differences in culture methodology (*e.g.* plate versus liquid culture) and in the substrate used for enzyme activity measurement. Other causes for concern relate to the apparent high specificity shown for oestradiol and closely related chemicals in some of these systems (*e.g.* Klein *et al.*, 1994) and the differences in response recorded for various putative anti-oestrogenic agents. Indeed, some models do not appear able to identify such chemicals on the basis of their co-administration with oestradiol (Wrenn and Katzenellenbogen, 1993; Kohno *et al.*, 1994). Other problems identified by some of the authors (*e.g.* Berry *et al.*, 1990; Wrenn and Katzenellenbogen, 1993) include interference with endpoint measurement through cell toxicity/lysis and the established problem of the limited ability of some chemicals to cross into the cell through the specialised cell walls found in yeast.

156. Given the wide variations in the methods used, it is not possible at present to identify a single, agreed methodology that can be recommended for formal adoption. However, so long as the basic limitations of the model are accepted, a transfected yeast cell expressing the human oestrogen receptor has much to recommend it because it has been well established as a sensitive and reliable model for a wide

range of chemical types. It is therefore considered that resources should be allocated to the optimisation and subsequent validation of such a model.

Androgen-related test systems

Human cell lines

- **Saos-2 and U2-OS human osteoblast cell lines**

157. During investigative studies on Saos-2 and U2-OS human osteoblast cell lines, Orwoll *et al.* (1991) successfully demonstrated the presence of androgen-binding sites in these cells by measurement of competitive binding studies on cellular fractions. Receptors were expressed at levels similar to those found in the normal human kidney, an established androgen-responsive tissue. Some inhibition by progestin was seen, however, and the metabolic status of the cells was not addressed. At present there is insufficient ground to confidently develop these models as a screen for androgenic activity. Nonetheless, the presence of androgen receptors in this type of cell raises questions as to the range of cell types that might be sensitive to androgenic or other sex hormone stimulation, and hence the potential difficulties in attempting to define sex hormone-disruptive activity for a chemical on the basis of *in vitro* assays using a very limited number of tissue types having known receptor profiles.

Animal tissues and primary cell screens

- **Mouse embryonic hypothalamic cell culture**

158. Beyer *et al.* (1994) studied variations in aromatase activity in cultured mouse embryo brain cells exposed to a number of oestrogens or progesterone and testosterone. In the model, day 15 embryo cells were cultured for up to six days in the presence of one or more of the test chemicals before assessment of aromatase activity, using a $^3\text{H}_2\text{O}$ formation assay employing radiolabelled testosterone. Immunohistochemistry was also used to visualise the aromatase-active neurones. This *in vitro* embryonic hypothalamic cell culture system was shown to be sensitive to androgenic stimulation and anti-androgenic inhibition and demonstrated differences in responsiveness between the sexes. This is unsurprising, given the key role sex hormones have in the development of the brain in mammals. The changes in enzymatic activity recorded were found to be closely correlated with actual physical changes in the development of the neurones.

159. This model was used as a research tool rather than a screen. It required technically competent staff working with relatively sophisticated equipment. In addition, the number of chemicals examined was very small and data presented suggested that the effect may be solely mediated by androgen-receptor interactions. Data on the metabolic capacity of these cells are not available, so it is possible that the system may be unresponsive to chemicals that require initial metabolic activation before interacting with androgen receptors or that act through other, non-receptor-mediated pathways.

Animal cell line screens

- **Transfected PC12 neuronal cells**

160. In a paper by Lustig *et al.* (1994), the effect of two days of exposure to dihydrotestosterone on neuronal cells derived from a rat pheochromocytoma was studied, following transfection of a human androgen receptor and a CAT reporter gene. The wild-type cell, PC12-WT, is known not to express androgen receptors, so low background interference could be expected. Several endpoints were examined in this investigative study, including androgen receptor expression, by Northern blot, cytosolic binding and histopathological morphology. The cells were found to display dose-dependent responses to the androgen investigated with a reasonable degree of sensitivity: from 10^{-10} to 10^{-7} M for the morphological endpoints. However, only one compound was tested in the study reviewed and the methodology was not considered to be sufficiently developed to warrant further consideration as a screening tool.

Insect cell line

161. Wong *et al.* (1993) demonstrated that an insect cell line (Sf9, derived from *Spodoptera frugiperda*) which had been transfected with a human androgen receptor was sensitive to androgenic agonistic and antagonistic chemicals. This paper focused on the interactions that occur between androgens, the androgen receptor and DNA. Hence the endpoint studied was DNA binding of the receptor. It would be of interest to investigate the responsiveness of this system to non-hormonal chemicals that have exhibited androgenic activity in other test systems. However, because of the methods used, this system is not considered suitable for use as a screening tool.

Yeast screen

162. Purvis *et al.* (1991) have published on the development of a series of yeast models containing a transfected human androgen receptor and the study of their sensitivity to dihydrotestosterone. In this system, Western blot and band shift assays were used to monitor cell responsiveness. Several transfectant strains were derived that were responsive to androgenic stimulation, with reproducible expressions being possible over a wide range (1400-fold). Clearly, the model as constituted would be unsuitable for adoption as it is insufficiently characterised, has only been tested on one chemical, and employs complex endpoints. However, given that the sensitivity of suitably transformed cells to androgenic stimulation has now been established by this work, and that oestrogenic-sensitive yeast systems with relatively simple endpoints have also been established and are considered of possible use as screening tools (see above), the potential may exist to develop a suitable androgen-sensitive yeast model. Reservations relating to such models have been discussed previously.

3.3.4 Cell-free tests for oestrogenic, androgenic or related hormones

Receptor-binding studies

163. Cell-free receptor-binding studies have been widely used in the mechanistic study of chemical:receptor interactions. These techniques are able to show whether a given chemical can bind with a particular receptor or not, with the inference that a high binding affinity would potentially result in marked biological activity of some type. Several limitations exist that severely restrict the role of such

techniques in chemical screening. These include: difficulties in distinguishing between agonistic and antagonistic receptor binding; a positive result may occur for compounds which have physical characteristics that would make it unlikely that they would normally enter the body or cell in the *in vivo* situation (this is a common problem with all cell-free systems); such systems do not address post-receptor interactions; and no account of metabolism or excretory processes, both of which may significantly influence the chemical receptor interactions *in vivo*, is possible. It may be possible to incorporate hormone-stripped auxiliary metabolic sources into such cell-free systems, but difficulties with non-receptor protein interactions would then be a potential problem. Further limitations include the inability to predict the ultimate effect which would be elicited, and the dependence of these tests on the assumption that interactions with known receptors will be the mechanism by which toxicity is mediated. It is thus questionable if such studies can currently play a significant role as a general screen, although they are undoubtedly of value during candidate selection for particular classes of chemical where processes have already been characterised, and in the detailed mechanistic investigation of chemicals following their identification as potential endocrine disruptors by suitable screening systems.

- **Microtubule (mt) polymerisation**

164. The effect of oestrogens on the polymerisation of microtubules was studied in a cell-free system derived from bovine brain tissue by Metzler and Pfeiffer (1995). They used diethylstilboestrol and a number of other steroidal oestrogens, mycoestrogens and lignans. The endpoint examined was percentage polymerisation of the MT, assessed by optical density. When the inhibitory effects of various compounds on MT assembly were compared with known oestrogen activities and binding affinities, no obvious correlation was found. Stilbene oestrogen's inhibited MT polymerisation regardless of whether they displayed high oestrogenic activity; conversely, powerful steroidal oestrogens failed to have any effect. It was thus apparent that the inhibitory activity of the stilbene derivatives was independent of oestrogenic activity. Although this method is of no value as a test screen for oestrogenic potential, it has shown that a chemical may have more than one mode of action or activity.

- **Oestrogen-2-hydroxylase (aromatase) activity**

165. Osawa *et al.* (1993) studied the activity of aromatase in placental microsomes. Further work by O'Reilly *et al.* (1995) demonstrated an inhibitory action of substituted androstenediones on the aromatase reaction. On the basis of the limited data available, this method appears to be a useful means of determining potential inhibition of aromatase, and thus of disruption of the formation of oestrogens from androgens. It may therefore be of some value in mechanistic investigations. However, this system has the usual limitations of this type of model and, given its limited development, it is not considered to be of proven benefit as a screening tool.

- **Monoclonal antibody enzyme immunoassay**

166. The ability to screen for 2- and 16 α -hydroxyoestrone (major metabolites of oestradiol) using monoclonal antibodies has been suggested as a possible diagnostic tool to detect disturbed oestrogen homeostasis. As such, it would have potential application to monitoring of humans and animal models *in vivo*. Further work is clearly necessary to quantify the relationship between this potential biomarker and exposure levels before it could be confidently used. If such a relationship could be established, the assay might have an important role in assessment of exposure and in assisting in the interpretation of *in vivo* test models. However, non-steroidal compounds such as phytoestrogens may interfere with the assays and, if

present in high enough concentrations in urine, could adversely affect the reliability of the assay. At present this system is insufficiently developed for further consideration.

- **Development of a marker of oestrogenic exposure in human serum**

167. A possible extraction and separation procedure to enable identification of endogenous oestrogens and xenoestrogens in human blood was proposed by Sonnenschein *et al.* (1995). This involved complex separation stages followed by bioassay using MCF-7 cells. The authors also discussed its potential use as a marker for exposure to breast cancer-inducing chemicals. Clearly this is not a screening test, but the methodology could conceivably be of use for exposure, metabolic and toxicokinetic evaluation.

3.4 RELEVANT *IN VIVO* SYSTEMS FOR POTENTIAL EFFECTS IN WILDLIFE

3.4.1 Animal developmental and reproductive studies

168. Many different non-regulatory developmental and reproductive study designs have been reported which, by their very nature, are unlikely to define the mode of action of the chemical agent (*e.g.* oestrogenic or androgenic agonistic or antagonistic agent). They do, however, permit an overall assessment of the likely impact upon reproductive performance of the chemical's action and act to alert one to the need for more detailed, mechanistic investigations. The majority of reviewed studies of this type employ fish and focus on their reproductive performance. In the majority of these, the zebra fish was the model of choice, thus underlining its suitability as a laboratory species. Most papers had in common the use of very similar preliminary studies to select suitable treatment levels for the larger, more complex studies that followed. Examples include an acute toxicity study on adult fish and an embryo/larval study using eggs derived from untreated parents. Details of the preliminary study used to set treatment levels in the Wester and Canton (1986) paper were not, however, presented.

169. Bresch (1982) reported on a testing strategy to investigate the reproductive performance of zebra fish following exposure to xenobiotics. Acute studies were performed using pentachlorophenol, Arochlor, nitrophenol and a number of metals, whilst chronic data were presented for cadmium. The method presented in the paper is open to criticism. The test strategy was protracted, and reproductive performance assessment included the testing of different concentrations on the same batch of animals. Although this limited the number of animals precludes the conclusive attribution of an effect to a given concentration because it may be a delayed effect from a previous (lower) concentration. This will confound data interpretation and result in inappropriate designations of "effect/non-effect" levels. In addition, a major endpoint used was spawning rate, which proved to be very variable and difficult to interpret despite the collection of considerable baseline data. The use of total number of eggs was also recommended as a suitable marker in this study, but in later work was found to be unreliable.

170. Landner *et al.* (1985) published a paper on a testing strategy designed to provide information on many aspects of reproductive performance using a battery of short-term tests. Zebra fish were again used. Aspects covered included acute (96-hour) toxicity in adult fish under semistatic conditions and changes in spawning rates following chemical exposure. The numbers of viable eggs produced from exposed parents, the hatching success, and the time from fertilisation to hatching were also assessed by visual monitoring of the eggs produced, with the larvae then being monitored for abnormalities until death from starvation. Additional studies were performed in which exposure commenced at day 2 of age. The larvae were then monitored for development and length of survival until death from starvation. Overall, the authors recommended adoption of the following in an assessment of fish toxicity: pre-exposure of adults for at least 14 days before assessment of spawning; assessment of survival and stress tolerance of offspring of exposed parents; and embryo/larval tests following exposure of the eggs and larvae of unexposed parents. The endpoints developed appeared easy to achieve and were sensitive and reliable. The benefits gained from continuing observation until death of the larvae by starvation rather than using a fixed duration would, however, require justification taking into account humane considerations.

171. In a study using the Japanese rice fish (Medaka), the long-term effects of hexachlorocyclohexane were studied by Wester and Canton (1986). Effects of exposure of adult for up to three months were compared to longer-term exposures (from one month of age or from the egg stage). Although including assessments of behaviour, gross appearance and bodyweight changes, this study

principally focused on a detailed histopathological examination of the fish, including ultra-thin sectioning and electron microscopy. The study did not include any assessment of reproductive performance or behaviour and is not considered suitable as a screening tool.

172. A study design reported by Bresch *et al.* (1990) overcame many of the reservations relating to the earlier 1982 Bresch paper. Zebra fish were again the model. The effects of chloroaniline were studied. In this design adult fish were exposed to the test chemical, and spawning capacity and egg viability were assessed at intervals over 21 weeks. Samples of eggs were then taken and allowed to develop, under conditions of continued chemical exposure, to adulthood, when egg laying and subsequent development of the F₂ generation were assessed. The benefit to be gained by continuing the study of the F₂ generation beyond the period required to reach sexual maturation is questionable, and testing would take over 30 weeks even if the modifications suggested by the authors were incorporated. This model used a through-flow exposure system which is likely to prove problematic where chemicals are only available in restricted quantities. Despite these significant reservations, it should be stressed that this multigeneration model was the only one of the reviewed fish studies that could be expected to detect morphological abnormalities in the F₁ and F₂ offspring with this test chemical. Clearly the resource, time and cost implications of the general adoption of such a protocol would be considerable, and it is impractical as a screening tool. However, it may be appropriate to consider development of an OECD multigeneration fish protocol for use in specific cases where a definitive study is justified. Alternatively, it would be of interest to investigate whether some extension to existing embryo/larval protocols would permit identification of such effects within a reasonably short time span.

173. Thus, although none of the models reviewed above can be considered as suitable for direct regulatory adoption, the various issues raised by these studies suggest that it may be appropriate to review current OECD fish study designs for possible incorporation of additional endpoints or to consider development of other specific designs, *e.g.* multigeneration fish studies.

3.4.2 Sex-reversal assays

174. Many non-mammalian vertebrate species have been shown to demonstrate plasticity of sexual development in response to chemical or other environmental agents. Many test methods based on this property have been reported. These tests have variously been developed to act as non-specific or specific hormonal identifiers.

175. Of the authors that do not ascribe particular oestrogenic or androgenic specificity to their models, Lance and Bogart (1992) used alligators whilst Elbrecht and Smith (1992) studied developmental responses in chickens. In the alligator model, eggs were aseptically injected with test material incubated at either the male-producing or female-producing temperature and allowed to develop until day 61 or 75. Fetuses were then removed, decapitated, and gonadal histopathology assessed. Three aromatase inhibitors, two antioestrogens, and antiandrogen, oestradiol and dihydrotestosterone were investigated using this model. Clearly this model is unsuitable as a screening system given the long study duration, the limited availability of alligator eggs, their high sensitivity to manipulation, and the gaps in knowledge on the basic physiological and hormonal processes involved in sex determination. The effects of aromatase inhibitors, testosterone and tamoxifen on sex determination were studied in the chicken model. Here, sex was provisionally determined on the basis of genotypically controlled feather colour followed by examination of the sexual structure of the cloaca, serum hormone levels and gonadal histopathology. This model uses a readily available species and appears feasible for laboratory use, despite being quite protracted. It responded to aromatase inhibitors, but did not appear to respond to chemicals with other activities. Overall, this model is considered of doubtful general applicability.

176. Bergeron *et al.* (1994) studied the sensitivity of turtles to oestrogenic stimulation. This was a simple *in vivo* test system in which eggs were spotted with environmentally relevant doses of PCBs or oestradiol and then permitted to develop for a further four weeks before assessment of gonadal sex and genital duct development by gross examination with subsequent histopathological corroboration. Thus the endpoints were well defined and did not involve complex analytical procedures. Negative aspects of the test include the large group size used, the duration of the experimental period (approximately seven weeks), and the limited availability of the test species. Given these considerations, this model would not be suitable for routine use.

177. Denton *et al.* (1985) used the sexually dimorphic poeciliid mosquito fish to study the potential androgenic activity of mycobacterially produced metabolites of soya bean extract. Although this was a very simple test system, in which female fish exposed to the test agent for up to two weeks were visually assessed for changes in gonopodial structure, in the laboratory it produced effects in a target species that corresponded to changes seen in wild populations exposed to xenobiotics. It has therefore confirmed that females of this species are capable of responding to androgenic stimulation by developing male characteristics within a relatively short time of exposure. Plasticity of secondary sexual characteristics in response to chemical exposure is likely to occur in other fish species, possibly including some of those already specified as suitable for use in the existing OECD fish guidelines. The factors controlling sexual dimorphism in specific species and the specificity and sensitivity of responsiveness, using a reference range of chemicals, would need to be established before any particular model could be relied upon to identify a particular hormonal interference mechanism. In a more demanding study design, Yu *et al.* (1993) demonstrated that exposure to an androgenic hormone implant will elicit masculinisation of the gonads of the amphibian (frog) tadpole at temperatures normally associated with the female-type development. This was shown by pathological examination, organ weight analysis, and endogenous sex hormone assay. The method permitted systemic exposure of an *in vivo* system during the early developmental stages of the life cycle. However, drawbacks include the need to employ specific metamorphic stages with consequent difficulties in animal availability, the long treatment period (three months), and poor control of dosimetry. In addition, ovarian masculinisation can be elicited by various non-sex hormone influences (*e.g.* temperature, hypophysectomy, hypothyroidism, and inhibition of steroidogenesis enzymes). This indicates that control is a multifactorial process and that the specificity of a test based on such a model would be low.

178. The various sex determination/reversal assays have been used to investigate various aspects of the systemic, *in vivo* activity of a range of xenobiotics on reproductive development. In the case of the Elbrecht and Smith chicken model, high specificity of response to aromatase inhibitors was demonstrated, which effectively limits its use as a general screening model. Ideally, it would be desirable to identify systems capable of detecting a wide range of sex hormone-disrupting activities (*e.g.* oestrogenicity, anti-oestrogenicity, androgenicity and anti-androgenicity). This would necessitate cross-comparison of the responsiveness of various test species and systems, using a battery of model chemicals. Other questions that would have to be addressed include dosage sensitivity and reproducibility, availability of the test species, and the test duration. Given the general conservation of hormonal and developmental mechanisms across species, it may be possible to define a small number of marker species. However, such systems cannot at present be satisfactorily defined.

3.4.3 *In vivo* vitellogenin induction assays

179. Plasma or tissue vitellogenin or surrogate markers have been extensively used in *in vivo* laboratory and field situations as an indicator of exposure to oestrogenic agents for a wide range of

oviparous species. The scientific basis for the selection of this protein as a biomarker for oestrogenic stimulation is well established from basic reproductive, physiological and biochemical knowledge, although in the case of some invertebrate species synthesis is known to be influenced by hormones, leading to the potential for stimulation of vitellogenesis by non-oestrogenic chemicals (Taylor *et al.*, 1991). In addition, the toxicological significance of the production of this protein in immature animals or adult males of responsive species, and its impact on reproductive effectiveness, have yet to be determined. Thus, although clearly a marker for the presence of an oestrogenically active chemical and hence of potential use in a screening test, this endpoint cannot at present be considered a toxic endpoint in itself.

180. A number of the papers reviewed used various surrogate biomarkers for vitellogenin production rather than measuring the protein itself (*e.g.* protein-bound phosphorus in Robinson and Gibbins, 1983; plasma calcium in Periera *et al.*, 1992). Some (*e.g.* Robinson and Gibbins) used apparently technically demanding procedures in measuring vitellogenin. Similar criticism may be applied to the vitellogenin-specific mRNA assay of Ren *et al.*, 1996. In contrast, assays using RIA or ELISA techniques to directly measure vitellogenin appear to have been widely established and have proved suitable for both field and laboratory experiments on a wide range of chemicals. These assays appear to be generally very sensitive and reliable, although Nimrod and Benson (1996) did see considerable variation in response between individuals when using a catfish species. It is unclear if this arose from assay method problems or is a characteristic of the species used.

181. The outstanding problems with vitellogenin measurement relate to lack of standardisation of the bioassay system and the study protocols in which they have been used, together with the highly species-specific responsiveness of the antibodies used to date. Clearly the first of these could be addressed by a validation/ optimisation exercise, and the incorporation of this parameter in existing test protocols on oviparous species may be worth considering. (Inclusion of a group administered both the test chemical and a known oestrogenic agent (*e.g.* oestradiol) might also permit monitoring for anti-oestrogenic activity or additive/synergistic oestrogenicity.) The work of Heppell *et al.* (1995) suggests that it might be possible to develop a widely responsive (if not actually universal) vitellogenin immunoassay. Further work by Tyler *et al.* (1996) [not reported in detail in this review document] studied the cross-reactivity of a carp (*Cyprinus carpio*)-vitellogenin radioimmunoassay using representative species from a number of families including the Clupeiformes, Cypriniformes, Salmoniformes, Siluriformes, Cyprinodontiformes, Gasterosteiformes, Perciformes, Pleuronectiformes and Scorpaeniformes. The assay was very reproducible, with the mid-point of the standard curve lying between 18 and 22 ng/ml, and good cross-reactivity was shown with various members of the cyprinids. Other families, however, showed little or no cross-reaction. Clearly further work in the area of cross-species reactivity is required to optimise performance, whilst Tyler *et al.* have suggested that adoption of an ELISA rather than a radioimmunoassay technique would be preferable for ease of use.

3.4.4 Additional hormonal endpoints

182. MacLatchy and van der Kraak (1995) studied the responses of the goldfish to intraperitoneal administration of the phytoestrogen β -Sitosterol in a short-duration (four-day) assay. Plasma hormonal levels were used as the endpoints and showed some evidence of a dose-related response in each sex. The authors suggested that the changes seen paralleled the actions of oestradiol on reproductive performance of the fish. These findings suggest that hormonal changes may represent an alternative endpoint to the use of vitellogenin screens.

183. Although there seems little justification for adopting this particular model as a screen, because this species is not routinely used in routine toxicity testing, it may be appropriate to consider the possible inclusion of blood hormone assays in the existing toxicity test protocols on ecologically relevant fish species such as those recommended by OECD ([Annex 1](#)). More work would, however, be needed to validate responses against a range of model chemicals, to further investigate dose-response relationships, and to define an appropriate sampling regimen. It should be remembered that the endpoints used do not *per se* provide mechanistic information and must therefore be interpreted with caution.

3.4.5 Invertebrate tests

184. In a paper by Baldwin et al. (1995) potential physiological and biochemical targets for oestrogenic stimulation were investigated in daphnids. Diethylstilboestrol was studied in acute and multigeneration studies. Survival, moulting frequency, growth and steroid biochemistry (using radiolabelled materials) were assessed. It was shown that changes in metabolic steroid metabolism could be elicited by chemical exposure but also showed clear responses in respect to many of the simpler endpoints. It is therefore considered likely that the current OECD designs are adequate to assess effects in daphnids. However, given the wide range of endocrine systems and reproductive strategies that occur across the invertebrate phyla, there remains a need to develop tests on a range of representative species. Selection of suitable candidate species is likely to first require basic research into comparative invertebrate endocrinology and physiology.

3.4.6 Imposex in molluscs

185. Following the discovery of changes in the sexual morphology of female gastropod molluscs in wild populations, significant scientific effort has been devoted to elucidating the mechanisms involved, including the development of a number of laboratory-based models. However, following the characterisation of the process and because the effect has to date been seen in only one class of compound (organotins), these methods (although of considerable scientific interest) are unlikely to be appropriate for use as general screening tools for other chemical classes.

3.5 RELEVANT *IN VITRO* SYSTEMS FOR POTENTIAL EFFECTS IN WILDLIFE

186. Few *in vitro* test models currently available have specifically focused on wildlife as opposed to human risk. That is not to say that human-orientated systems are not of assistance in assessing the potential ecological impact of a chemical, but that the availability of models reflects the historical focus of concern. Systems that are solely directed at wildlife monitoring include, for oestrogenic potential, vitellogenin induction in trout hepatocyte cells and receptor binding models in the sea trout and turtle. In addition, the detection of potential androgen stimulating chemicals has been investigated using a carp testes model.

187. An *in vitro* vitellogenin induction assay capable of detecting oestrogenic chemicals was initially developed by Pelissero *et al.* (1993). The test system, using primary liver cells obtained from rainbow trout, was relatively rapid, taking between eight and 12 days depending upon the exact protocol used.

Later workers generally adopted a two-day exposure period, which was said to offer the nearest achievable metabolic similarity to the *in vivo* situation. Vitellogenin measurement was achieved by radioimmunoassay. Chemicals investigated using such models included oestradiol, diethylstilboestrol, oestradiol, testosterone and metabolites, progesterone, cortisol, phytoestrogens and alkylphenols. There was also an assessment of the inhibitory activity of tamoxifen. The methods generally restricted collection of hepatocytes to male fish in order, presumably, to minimise the chance of any expression of vitellogenin in the untreated (control) cultures. The systems were responsive to a large number of chemicals and were generally found to give good sensitivity and specificity although similarities in responsiveness were observed between a number of phytoestrogens and some androgenic or progestational agents. It was not possible to extrapolate directly to *in vivo*. These limitations raise some questions on how the results could be interpreted if used in a screening battery. Given the potential ease of use of some of the *in vivo* vitellogenin assays (see Section 0), the benefits to be gained from adoption of an *in vitro* vitellogenin model may be questionable.

188. A number of other model systems were reviewed. Using cytosolic extracts of spotted sea trout livers, Thomas and Smith (1993) quantified oestrogen receptor (ER) interactions within a cell-free system using radiolabelled hormones. The model was shown to detect oestrogenic and anti-oestrogenic activity. Because this is a cell-free system, results are difficult to extrapolate to the *in vivo* situation and this model is subject to the same constraints and criticisms as the human-relevant models. The inference that can be made from this work that there may be considerable species specificity for receptor interactions does, however, have important implications for the development and use of models. Ho *et al.* (1988) developed a method that overcame many of the previously experienced problems in detecting cytosolic oestrogen receptor binding in the turtle, but that remained technically demanding. Overall, the models considered are not suitable as screening tools although the methodology may be of use in specific mechanistic investigations. Although not intended for use as a test system for sex hormone-disrupting chemicals, the *in vitro* carp testicular cell model developed by Yu and Lin (1986) was shown to give a dosage-related androgenic response to stimulation by gonadotrophins and varying susceptibility to stimulation of androgen synthesis and release by exogenous chemicals (theophylline versus xanthine). The biological significance of this endpoint is, however, unclear.

189. It is therefore considered that none of the wildlife *in vitro* tests reviewed, with the possible exception of the trout liver culture system for vitellogenin induction, can be recommended for further progression.

3.6 RECOMMENDED NON-REGULATORY MODELS

3.6.1 *In vivo* systems relevant to humans

190. Given the current state of scientific knowledge, endocrine disruptive activity can only be definitively ascribed on the basis of *in vivo* data. Despite requiring the use of live animals, the potential scientific benefits of an *in vivo* over an *in vitro* screening model have been widely established. For example, Galey *et al.* (1993) noted that there were circumstances, such as when testing a fodder crop for oestrogenic activity, in which complex analytical equipment may not be readily available, preventing an analytically based assessment, whilst *in vitro* systems are often unsuitable because of the need for specialist knowledge or equipment or because of such systems' absence of metabolic capability. The benefits of an *in vivo* test model were further supported by O'Connor *et al.* (1996), whilst Jordan *et al.* (1985) and a recent European workshop (EC, 1997) stressed the need to evaluate any data from an *in vitro*

system in the light of findings from a suitable *in vivo* model. This is necessary in order to overcome intrinsic deficiencies in the *in vitro* situation, *e.g.* inadequate provision for metabolism, alternate modes of action, homeostatic mechanisms.

191. On the basis of the current review, two *in vivo* models have been identified that would permit screening of chemicals for oestrogenic agonistic or antagonistic activity. A further model is suggested to screen for effects on the androgenic system. Although each of these models has a long history of use, no definitive study design has been agreed between workers and, for each model, it will be necessary to develop a definitive design and validate its sensitivity and specificity against a range of reference chemicals.

192. Suitable models for screening of oestrogenic hormone-disruption are the rodent uterine and vaginal assays, with the rat being the species of choice (for the uterotrophic assay, the rabbit has been identified as a suitable alternative should the use of rodents be impractical for a given chemical). Depending on the study design adopted, both these models can screen for agonistic and antagonistic agents, although the most appropriate treatment regimens will require definition. Other aspects requiring clarification are the relative specificity and sensitivity of each test. Certainly vaginal keratinisation is highly specific, having been demonstrated only for oestrogenic agents, whilst there is evidence that the uterotrophic response may be elicited by a wider range of hormonally active agents. In contrast, some workers believe the uterotrophic response to be the more sensitive of the two. Many endpoints have been investigated at different times for both uterotrophic and vaginotrophic models. Of these, uterine weight and vaginal morphology (by smear/wash or histopathological examination), respectively, appear the most efficient whilst, in immature animals, time of vaginal opening has also been suggested as a suitable developmental marker. The use of vaginal smears as a screening tool would have advantages over the uterotrophic assay (including rapidity and the possibility of repeat sampling from individual animals), but a definitive recommendation cannot be given in the absence of contemporaneous comparative data. O'Connor *et al.* included vaginal washing/slide examination in a test battery otherwise heavily reliant on uterine endpoints. The authors considered that their multiple endpoint system would be a reliable, quick and relatively low-cost screening test. However, several of the endpoints used were variable and showed low specificity. This protocol is also considered too expensive for use as a screen. It is not clear from the literature if it is necessary to use ovariectomised animals in these test systems, as immature animals have been found satisfactory by some workers; this would reduce the effort and expertise required for the tests and minimise stress on the animals. These aspects should be investigated during any validation process.

193. The recommendation of an *in vivo* androgen-related assay of relevance to humans is more difficult. The most promising tests are those rat models that utilise changes in accessory sex organ weights, particularly the prostate, as the endpoint, although the specificity of the endpoint requires clarification. Designs using the change in prostate weight of castrated rats could certainly be used as a marker for both androgenic agonistic and antagonistic activity. Other related endpoints, such as prostatic DNA and RNA or ventral prostate marker enzyme activities (*e.g.* ornithine decarboxylase), have been suggested as alternative or supplementary markers and may have a role in some instances.

194. It remains to be established if tests on post-weaning animals are sufficient to act as screens for subtle developmental effects that may be elicited from exposure during pregnancy or lactation. Some models, including the mouse prostate models for oestrogenic substances, overcome this potential concern to some degree by utilising a prenatal exposure period, but further basic research on this aspect will be required.

195. An additional point relates to the apparent inverted-U dose response relationship observed by vom Saal *et al.* (1997) following prenatal exposure of male fetuses to oestradiol or diethylstilboestrol and measuring of prostate weight and other endpoints in adulthood. Although it is well known in endocrinology that low concentrations of a hormone can stimulate a tissue whilst high concentrations can have opposite effects, it remains to be seen whether this phenomenon is likely to be a common occurrence for other endpoints and for other endocrine-disrupting substances, but it may have important implications for dose selection and testing in such studies.

3.5 *In vitro* systems relevant to humans

196. As discussed above, endocrine-disruptive activity can only be definitively ascribed on the basis of *in vivo* data. The most promising use of the *in vitro* methods is in detailed study of the mechanisms of action within a given chemical series where there is some pre-existing knowledge of pharmacological activity. The methods may also have value in the future as screens for sex hormone-disruptive potential, but there are many problems to surmount. Perhaps the greatest concerns are:

- Difficulties exist in interpreting the significance of *in vitro* findings to *in vivo* situations because of the many factors that influence the outcome in intact animals and man (*e.g.* dosimetry, absorption, metabolism, excretion rates, bioaccumulation potential, pharmacokinetics and pharmacodynamics).
- Given the recent scientific debate concerning apparent synergistic effects of combination chemicals in a yeast-based assay, it is clear that reproducibility of results is a potential concern. Consequently, such test systems and conditions should be clearly defined and widely available to enable confirmatory testing to be carried out.
- Test endpoints are dependent upon interactions with a given receptor structure or response element, and difficulties exist for many such systems in distinguishing agonistic from antagonistic activity.
- Existing *in vitro* systems only provide information on chemical interactions with known receptors and cannot address the potential, but currently unidentified, mechanisms that may exist within an intact organism (*e.g.* the oestrogen-sensitive yeast models used what was believed to be the only human oestrogen receptor). With the recent finding that there are multiple receptor forms (Pink *et al.*, 1995; Watson *et al.*, 1995; Mosselman *et al.*, 1996; Pink *et al.*, 1996), questions must be raised as to the suitability of existing *in vitro* models to act as satisfactory surrogates for the intact animal, *i.e.* complimentary *in vivo* data are likely always to be necessary.
- The current absence of a satisfactory metabolising system for use in *in vitro* established cell line or cell-free systems limits the usefulness of such receptor-mediated assays. Several yeast-based assays offer some degree of metabolising competence, but this will be different from that found in animals, and problems can also be expected to occur for some chemicals in yeast because of difficulties in bioavailability due to poor translocation across the cell wall.

197. *In vitro* models may be divided into several major classes: tissues or primary cells of human or animal origin; established cell lines derived from human or animal tissue; and yeast-cell based assays. Established cells and the yeast cells may be modified by the use of transfection techniques to incorporate

specific receptor or reporter structures. Cell-free systems and structure-activity-relationship (SAR) mathematical models have also been developed. Clearly, each of these categories will have generic strengths and weaknesses in addition to the specific attributes of the individual test models. *In vitro* models can potentially provide data of value to human risk assessment and, by inference, may also contribute to the assessment of potential risks to wildlife species. However, in some cases the model or the endpoint examined will have little relevance to man, being focused instead on wildlife (for example, in the case of the fish liver vitellogenin assay).

198. The use of human primary tissue models is not considered to be practicable. Although human tissue (*i.e.* blood) is routinely collected for *in vitro* tests for mutagenicity (*e.g.* human lymphocyte test), the types of human tissue required for investigation of sex hormone-disruptive activity would not be available as readily. Relevant tissues would only be available when removed as part of the treatment of individuals suffering from certain disease conditions, when the “normality” of any tissue taken would be open to question. In addition, such tissue would be highly likely to pose significant health risks to workers. Other problems generic to the use of primary cells include the difficulties in maintaining a constant cellular biochemistry and physiology over any significant period (unless this is maintained, there will be a significant loss in predictive power) and difficulties in translating the significance of any effects seen to the intact organism.

199. From the review of the available models, none of the animal primary tissue or cell culture systems were considered to be sufficiently developed for use, or to have significant enough benefits over the use of established cell lines, to warrant further consideration. No suitable screening methods were identified among the cell-free models, although the potential use of some of these methods for mechanistic investigations was recognised.

200. Given the many limitations on the use and scope of *in vitro* models, it is clear that such models are not yet sufficiently developed to be considered as the sole basis for regulatory action. Indeed, it is questionable whether this goal will ever be achievable. However, some models appear of potential value and, as such, should be subject to further development and validation as potential future screening tools for use in a regulatory context. In the interim, it is likely that these tests will be of assistance in provisional (non-regulatory) screening during candidate chemical selection or in detailed mechanistic studies on particular chemicals.

Oestrogen-responsive models

201. Models sensitive to oestrogen which appear to warrant further development, cross-comparison and validation are the Ishikawa and MCF-7 cell lines and the yeast assay. In order to be accepted as regulatory screens, it is important that any testing strategy takes account of the various limitations and difficulties associated with these assays. Particular concern has been expressed over the existence of multiple clones among human cell lines, with resultant inconsistency of response. For example, marked differences in sensitivity have been established between different passages and sub-clones of MCF-7 cells by Villalobos *et al.* (1995). Despite such problems, the volume of data available on MCF-7 cells suggests that they should be included in further development programmes, even if only to provide a baseline comparator. It is possible that, with further study, similar difficulties will be found in many other lines. It will thus be necessary to identify suitable sub-strain(s) with defined characteristics (such as stability and sensitivity), whilst the benefits of wild type versus transfected cells will require clarification. As an example of the potential problems that may exist with *in vitro* assays, a paper published by Arnold *et al.* (1996a) indicated that various chemicals, when tested in binary combinations in a yeast-based oestrogen

assay, showed up to 1000-fold greater response than the effect of either chemical alone (*i.e.* synergistic action). Because of the interest surrounding the potential implications of this work, other groups attempted to reproduce the data by repeating the experiment, but without success (Ramamoorthy *et al.*, 1997; Ashby *et al.*, 1997; Gaido *et al.*, 1997). Subsequently the original paper was withdrawn, as the authors were themselves unable to replicate their own findings (McLachlan, 1997).

Androgen-responsive models

202. Recommending a suitable *in vitro* androgen assay is difficult, perhaps because these assays' reported use in the testing of chemicals has, to date, been limited. Further development of the yeast assay for androgens is suggested, because responsiveness to this hormone has been shown and much is known about this yeast from previous studies. By the same token, the same criticisms and limitations would apply to such a test as for the oestrogen assay using yeast.

Alternative approaches

203. The use of structure-activity relationship (SAR) studies for oestrogens and androgens may be a promising additional avenue for continued research. However, as detailed in Section 0, there are currently unanswered questions on hormone receptor structure, function and interactions, and receptor sub-types, and there are inherent difficulties in extrapolating from a model to an *in vitro* and, ultimately, to the *in vivo* situation. Generalised use of SAR models as screens for novel chemicals should therefore be regarded as a future goal rather than a practical current possibility. Further basic research to address the unknowns and the continued development and refinement of SAR models are recommended.

Assay development

204. For any assay to be validated, precise definition of the model and the development of a standard set of parameters to measure reproducibility will be necessary. This would be aided by establishing the system's responsiveness to a reference set of chemicals for the appropriate range of activities. In any evaluation of tests using cell lines, stock uniformity is possibly the most important variable that could affect reproducibility. Thus, before adoption as a formal (acceptable for regulation) test, adequate characterisation of the cell line would be necessary and the test design would have to address factors such as drift in the responsiveness, sensitivity and specificity of cells, so as to ensure that variations in response did not occur between laboratories or over time at a single laboratory.

3.6.3 *In vivo* tests relevant to wildlife

205. Information from tests conducted on mammals (generally rodents) to investigate toxicity and sex hormone-disrupting activity are principally intended to assist in predicting a chemical's potential hazard to humans. However, the data thus derived will contribute to the understanding of a chemical's activity profile and will assist in understanding its potential impact on wildlife. Many aspects of the physiology and development of non-mammalian species nonetheless cannot be addressed using mammalian models.

This is particularly true for invertebrate taxa where markedly different physiological and endocrine systems exist. Given the very limited range of relevant non-mammalian OECD tests currently available, it can be appreciated that there is an urgent need to increase the chance of detecting unusual or novel activities by employing a wider range of ecologically relevant species. As noted in the discussions on future research needs, however, there is currently a lack of basic knowledge on which to base the selection of suitable marker species. In developing such tests it must also be remembered that whilst human risk assessment is based on assessing the potential risk to the individual, the basis for ecological risk assessment generally focuses on determining potential hazards to wildlife populations. Notwithstanding such aspects, the following models or endpoints were identified as potentially suitable for further development:

206. Radioimmunoassay or ELISA techniques to measure vitellogenin in a range of oviparous species should be developed and the potential for development of a universal antibody should be clarified. A vitellogenin assay would offer a rapid, sensitive screening tool to detect oestrogenic exposure in both laboratory and field situations, although questions remain as to the toxicological significance of abnormal expression of this protein in the absence of other changes of toxic importance. There is some evidence that other egg proteins may prove even more sensitive markers of oestrogenic activity (*e.g. zona radiata* protein in Atlantic salmon; Arukwe *et al.*, 1997). However, the relative benefit of this alternative marker in other situations remains to be clarified. At present there is no equivalent marker to vitellogenin that could be used as an assay to detect androgenic exposure. Funding of basic research to identify and develop such a marker should be considered. Until such a marker is developed, the robustness, sensitivity and reproducibility of fish models able to detect sex hormone-disrupter activity through changes in secondary sexual morphology may warrant investigation.

207. It is possible that it would be beneficial to include hormonal assays in fish toxicity study designs, as appropriate, or to develop a suitable "stand alone" assay, whilst there is some evidence that a multigenerational fish study design might be of value in elucidating the activity of a chemical in some circumstances. It is, however, recognised that such a prolonged study would not constitute a screening tool.

3.6.4 *In vitro* relevant to wildlife

208. Few of the *in vitro* test models currently available have specifically focused on wildlife as opposed to human risk. That is not to say that human-orientated systems are not of assistance in assessing the potential ecological impact of a chemical, but that the current availability of methods is a reflection of previous historical focus. On the basis of the review, none of the wildlife *in vitro* tests, with the possible exception of the trout liver culture system for vitellogenin induction, is suitable for further progression.

4. POTENTIAL CHANGES TO CHEMICAL TESTING PROCEDURES

4.1 CHEMICAL TESTING REQUIRMENTS AND STRATEGIES

209. The range of tests used on a novel chemical will vary greatly with a number of factors, including intended use, quantity to be produced per annum, etc. Particularly in the case of chemicals for agrochemical or other pest control applications and those for medical or veterinary use, a wide range of toxicity studies addressing subchronic, chronic, reproductive and possibly carcinogenicity endpoints are routinely conducted. The range of mammalian species used will also include various rodent species plus others, such as the dog or possibly a non-human primate (the selection of species for use has historically been based upon the similarities that exist between their physiological and biochemical systems and those of humans, together with considerations relating to availability, practicality for use in laboratory situations, and cost considerations). Chemicals of these classes will also be subject to additional testing for ecotoxicological hazard, thus further expanding the knowledge base on their activity profiles. Pragmatically, it is reasonable to assume that any inherent vertebrate endocrine-disruptive potential is likely to have been identified for an existing chemical that has been subject to such a full hazard identification process, although this may not be the case for potential activity in invertebrates. A number of reservations in respect of this position should be noted:

210. The traditional approach to toxicity testing has been to conduct tests using a number of dose levels ranging from overtly toxic to non-toxic in order to identify a no-observable-adverse-effect-level. It has been suggested that endocrine disruption may not follow the traditional dose-response pattern and that special consideration should be given to choice of dose levels in tests, although the reproducibility and frequency with which this phenomenon occurs remains to be clarified for different substances. Nonetheless, in order to identify true endocrine-disrupting activity it is necessary to establish that any disruption arises as a result of a primary action on the endocrine system rather than occurring secondarily to other (overt) toxicity;

211. Differences may exist in the basic endocrinology, biochemistry or physiology of routine test species compared with the vast array of species found in the wild. Also, effects may be restricted to specific susceptible life-stages that are not routinely assessed. Therefore, there is a need to extend our understanding of the comparative endocrinology of different animal taxa (including invertebrates) and their susceptibility to endocrine disruption, and then to consider the possible need for the development of additional test models.

212. In the case of the existing chemicals used in pharmaceutical or agrochemical/pest control applications, it is therefore unlikely that additional testing will be required unless the chemical is suspected of possible sex hormone-disrupting activity on the basis of chemical class and structure, mode of action, existing toxicity data, or reports of effects in use or following environmental exposure. If necessary, existing experimental data should be reviewed for indications of possible effects suggestive of hormonal disruption and, where applicable, suitable screening tests to identify potential activity should be considered to identify priority chemicals for fuller reassessment, re-testing or detailed mechanistic study.

213. In the case of novel chemicals intended for pharmaceutical, agrochemical or pest control use, the opportunity exists to focus attention on the sex hormone-disrupter issue during the chemical selection and development process and to collect additional relevant data, where appropriate. Several endpoints have been identified as suitable for proposing for use in, or for introduction into, the existing OECD Test

Guidelines. Such modifications are likely to strengthen the ability of the existing test models to identify sex hormone-disrupting activity for vertebrate endocrine systems, although there remains a need to develop relevant tests to assess effects on the endocrine systems of invertebrates.

214. In the case of existing and novel chemicals of non-pharmaceutical or agrochemical/pest control categories (such as industrial chemicals), the adequacy of existing data requirements to identify sex hormone-disrupting activity may be questioned. In some cases it is likely that further testing of existing chemicals will be warranted, whilst for new chemicals the introduction of additional screening methods is considered advisable. It is assumed that definitive classification of a chemical as a sex hormone-disrupter will require detailed mechanistic studies including *in vivo* data. Any decision to increase the data requirements for these or other chemicals will require extensive debate.

215. A tiered hazard identification strategy applicable to all types of endocrine disrupters was proposed at the SETAC-Europe/OECD/EC EMWAT Workshop on Endocrine Modulators and Wildlife: Assessment and Testing (Tattersfield *et al.*, 1997), held in the Netherlands in April 1997. This scheme is presented in Figure 1. The scheme is designed to identify whether a substance has the capacity to cause endocrine modulation (used interchangeably with endocrine disruption in the report) and whether a risk assessment should be performed. During this process, aspects such as anticipated exposure levels and benefits would be weighed against the potential hazard posed by the chemical before a final decision on production and use limitations is reached. It is anticipated that the risk assessment processes applicable to endocrine disruption will require review in the light of the rapidly increasing scientific knowledge in this area of research. Therefore, only the initial stages of this tiered testing strategy (boxes one to six) will be discussed in brief here. The reader is referred to the EMWAT document (Tattersfield *et al.*) for a more detailed description of the scheme.

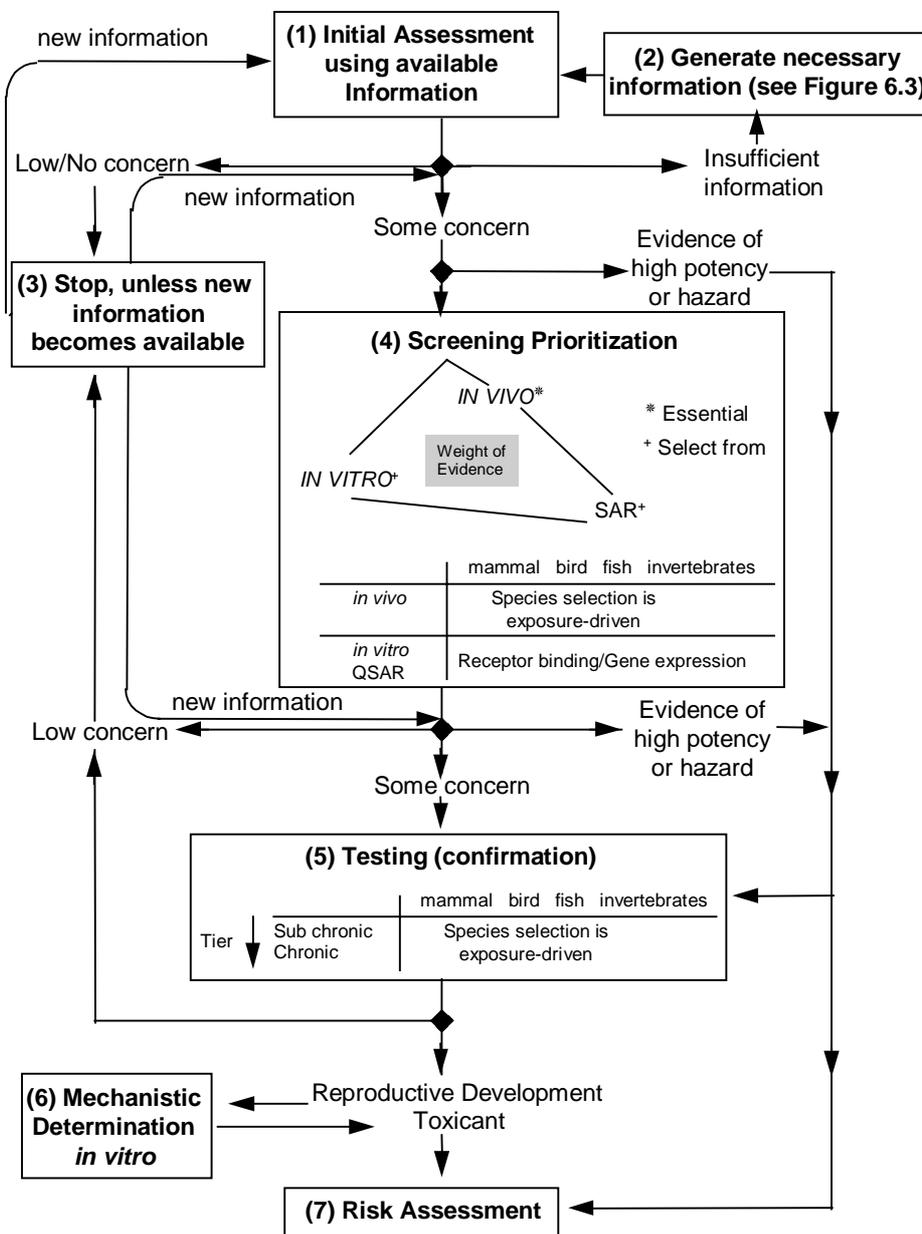
4.1.1 Initial assessment using available information

216. The testing strategy for any given chemical will depend upon the amount of relevant information available. All existing data on a substance should be assessed at this pre-screening stage, including:

- data on production and use of the substance;
- environmental monitoring data (including release data in the terrestrial, aquatic and air compartments and potential for biodegradation and bioconcentration);
- existing ecotoxicity data;
- predicted effects based on structure-activity relationships;
- data on likely impurities, metabolites and breakdown products;
- existing monitoring or field data indicating evidence of endocrine effects (this data would have a significant impact on the prioritisation of substances for evaluation).

Figure 1

Proposed hazard identification scheme for the determination of potential endocrine modulating effects of new and existing substances (taken from Tattersfield *et al.*, 1997)



Data on a substance that indicated a more significant toxic mode of action (*e.g.* mutagenicity) would give it low priority regarding endocrine modulation effects, as the regulatory process would be driven by the most toxic activity.

217 In order to assist in the assessment of the information described above and to aid prioritisation, a so-called “Grey Scale” has been suggested to enable a qualitative categorisation of fate and effects (Tattersfield *et al.*, 1997). Such a scale may also aid in the identification of data gaps, which could then be generated and fed back into the initial assessment. Professional judgement would then be used to

assess the information and determine the extent of concern regarding potential endocrine-mediated effects in man or the environment. The important factors which suggest a substance may be of little or no concern include the following:

- It is unlikely to enter the environment.
- There is a more toxic mode of action which is of greater concern.
- There are particular structural features which exclude the likelihood of biological activity (*e.g.* very high molecular weight).

218. If it is concluded that a substance is of very low or no concern, then progression of the substance through the assessment scheme is halted until such time as new information becomes available to suggest otherwise. If, however, there is not enough information on which to assess the level of concern of the substance, it proceeds to the screening stage. Alternatively, the concern resulting from the initial assessment may be sufficient to proceed directly to testing, or risk assessment.

4.12 Screening/ prioritisation

219. The EMWAT strategy considers a short-term *in vivo* screen indicative of potential effects on reproduction and development to be essential for the screening of substances for potential endocrine modulating effects, as such a system incorporates metabolism and all potential modes of action (Tattersfield *et al.*). Data from *in vitro* assays and SAR analysis are considered to be useful at this screening level, for example in the elucidation of mechanisms of action, but at the current state of development of such models and with questions surrounding their validity and predictability, a negative result in such models would not be taken as definitive and would require confirmation of a lack of endocrine modulating effects by an *in vivo* system.

220. There are several important points in the selection of *in vivo* tests for screening purposes:

- Testing should be simple, rapid, relatively inexpensive, and should measure endocrine endpoints.
- Species selection should be determined by the potential routes of exposure.
- Testing should be carried out over a wide range of doses to account for different types of dose-response relationship.

221. Such *in vivo* tests are under development as rapid screens in mammals, birds and fish, but that there is presently a lack of available methods for invertebrate species (Tattersfield *et al.*).

222. The level of concern regarding a particular substance will rely on judgement and will depend on the weight of evidence from all the screening data taken together, with the greatest weight being given to *in vivo* data, followed by *in vitro* data and finally SAR data. A decision will then have to be made, on the basis of this data, whether to discontinue (unless new information becomes available), to continue to further testing or to go directly to risk assessment. However, it is not currently possible to define the triggers for these different decisions but only to conclude that a substance is of low or some concern (Tattersfield *et al.*).

4.13 Testing

223. It is suggested that there are two tiers to confirmatory testing; subchronic, which should allow substances with potentially different mechanisms of action to be integrated with reproductive and

developmental endpoints, and chronic, which involves definitive multigeneration studies. Similarly to *in vivo* screening, the selection of species should be driven by exposure considerations. The range of doses selected will depend on the *in vivo* screening data. The reader is referred to the EMWAT document (Tattersfield *et al.*) for a detailed description of *in vivo* testing strategies relevant to wildlife species (see Executive Summary in Annex 4).

224. If, as a result of testing, the substance has no effects on development or reproduction, no further action is necessary as it poses no potential hazard as a developmental or reproductive toxicant. If effects are seen, a risk assessment should then be made on that substance. Once a substance is confirmed as a reproductive or developmental toxicant, a mechanistic characterisation should then be performed using *in vitro* test systems to help to elucidate whether the substance is acting via mechanisms involving endocrine disruption.

225. Specific details relating to the risk assessment process for endocrine disrupters can only be developed when the procedures involved in hazard identification are developed, agreed and accepted (Tattersfield *et al.*, 1997).

4.2 EXTENSIONS TO EXISTING REGULATORY STUDY DESIGNS

226. When considering the hazard assessment process for mammals (and by inference humans), a promising approach appears to address gaps in the existing guidelines by including additional endpoints of relevance to sex hormone-disruption. In the review of the OECD Test Guidelines, a number of such additions were identified. There are plans to address several of these gaps in forthcoming revisions. Examples include: extension of organ weight analysis and histopathology of the gonads and accessory sex organs; guidance on best practice for pathological processing; detailed pathological examinations of offspring; blood hormone level monitoring (with guidance on sampling regimens); detailed assessment of spermatogenesis and/or sperm morphology, number and activity; vaginal cytology to monitor oestrus cyclicity; enhanced monitoring of physical and behavioural development and learning/memory functions; and, possibly, assay of accessory sex organ secretory products. The OECD guidelines to which such proposed enhancements could be applied are summarised in Table 4. In order to maximise the benefit to be gained from such extension, it would be advisable initially to focus attention on enhancing the subchronic tests because it is anticipated that these would be used on the widest range of chemicals and would provide early warning of the need to modify subsequent studies on particular chemicals to investigate potential endocrine disruptive activity.

227. A major concern is the limited scope of the current OECD Test Guidelines. In particular, consideration should be given to using a wider range of taxa and endpoints. Multigeneration studies in fish, birds and other key groups should be considered to develop and validate biomarker responses and other endpoints in subchronic tests. The limitations of existing OECD designs for fish and birds are clearly apparent, although a detailed review of avian reproductive testing has been undertaken by OECD and SETAC (OECD, 1996) and that the two relevant OECD guidelines, 205 and 206, are currently undergoing a revision process. Similarly, a number of new test designs for fish are under development which would be anticipated to improve the evaluation of non-mammalian vertebrates. There is an outstanding need to establish additional designs predictive for invertebrate groups, especially with regard to endocrine, developmental and reproductive systems having no mammalian correlate. It is, however, recognised that basic research may first be necessary.

Table 4**Proposed changes to existing OECD Test Guidelines**

Endpoint	Test Guideline
Extension of organ weight analyses on gonads and accessory sex organs	206, 407, 408, 409, 412, 415, 416, 421, 422, 452, 453 (toxicity phase only)
Extension of histopathology on gonads and accessory organs/provision of guidance on appropriate techniques	206, 407, 408, 409, 412, 416, 451, 452, 453
Inclusion of detailed pathological examinations of offspring	415, 421, 422
Inclusion of sex hormone blood level assay/provision of guidance on appropriate sampling regimens	407, 408, 409, 412, 415, 421, 452, 453
Investigation of accessory organ secretory products	407, 408, 409, 412, 415, 421, 422
Detailed investigation of spermatogenesis and/or sperm quality	407, 408, 409, 412, 416, 421, 422, 451, 452, 453
Monitoring of oestrus cyclicity	407, 408, 409, 412, 416, 451, 452, 453
Enhanced physical/behavioural monitoring of offspring	415, 416
Enhanced learning/memory monitoring of offspring	415, 416

228. Before adoption, additional endpoints should be assessed to ensure their sensitivity as markers of endocrine disruption, whilst the relative benefit of using additional endpoints in existing guidelines as opposed to introducing simple “stand alone” screening methods for sex hormone-disrupting activity should be considered.

4.3 POTENTIALLY SUITABLE NON REGULATORY TEST METHODS

229. Given the current state of knowledge of endocrine disrupter mechanisms, *in vivo* models are considered preferable to *in vitro* ones. Review of the available non-regulatory test methods has identified

two human-relevant models capable of detecting oestrogenic modulation and one model that is suitable for demonstrating androgenic effects that might be suitable for use as screening tests. A number of suggestions for possible wildlife-focused studies are also made. Some *in vitro* models which might be developed and validated have also been identified (Table 5).

230. Screening models for oestrogenic agonistic or antagonistic activity are either the rat vaginotrophic or uterotrophic assays. Before a decision can be reached as to which is preferable, a number of aspects need to be addressed, including: the relative sensitivity and specificity of each assay; confirmation that vaginal cytological assessment (by smear or wash procedures) and uterine weight measurement, respectively, are the most suitable of the available endpoints for each assay; and assessment of the relative benefits of using ovariectomised or immature animals. Following clarification of these points, it should be possible to confidently develop a precise protocol for consideration as an OECD design.

231. The most promising *in vivo* approach to monitoring androgenic agonistic or antagonistic activity is considered to be measurement of accessory sex organ weight (particularly that of the prostate) of castrated rats, although the specificity of this endpoint may require confirmation. Using a different study design in mice, it is also possible to study the effects of oestrogenic agents on prostate weight and other related endpoints.

232. Additional information on other *in vivo* pharmacological assays to detect hormonal activity which might prove of value during development of suitable screening methodologies is provided in a review by Edgren (1994).

233. Males of many oviparous species have been shown to express vitellogenin in response to oestrogenic stimulation. This activity can be modified by use of appropriate antagonistic agents. Assay of this protein in such species could therefore be a valuable tool in wildlife monitoring and potentially permit the use of such animals in screening assays, although in the case of some invertebrate species potential interactions with other hormone systems must be recognised. Further refinement and validation of the radioimmunoassay or ELISA techniques used are necessary and should include investigation of the possible development of a widely applicable (“universal”) test kit. There are some data suggesting that types of *zona radiata* proteins (types of egg protein) may be more sensitive than vitellogenin as a marker of oestrogenic stimulation. However, additional data in a wider range of oviparous species are required before tests using this alternative endpoint could be suggested as the preferable marker for adoption. At present there is no equivalent marker available to detect androgenic modulation. Until such time as one is identified, it may be appropriate to investigate the use of changes in secondary sexual morphology shown by some fish species in response to exposure to sex hormone-disrupting chemicals. It will, however, be necessary to characterise the factors controlling such responses and to determine the sensitivity and specificity of the models.

234. Although they are clearly not initial screening tests, there is a need for multigeneration study designs in a range of species to facilitate the study of subtle effects of chemicals on reproductive processes (for example, multigenerational fish studies were addressed during the preparation of this review).

235. At present it is not possible to recommend adoption of any of the human-focused *in vitro* assays as a regulatorily acceptable model because of the various limitations and difficulties inherent in current designs. Aspects requiring resolution include: the dependence of *in vitro* model endpoints on specific receptor or response element interactions which may not mimic *in vivo* modes of action; the inability of some test systems to distinguish agonists from antagonists; the fact that existing *in vitro* models lack

satisfactory metabolic systems or may show only limited chemical uptake; the need for the significance of the *in vitro* findings to be extrapolated to the intact organism, where absorption, metabolism, excretion or bioaccumulation may play critical roles in determining activity. There is a need to establish the predictability and sensitivity of such models against an appropriate “gold standard” *in vivo* methodology. Further development is, however, strongly encouraged. In particular, cross-comparison and validation of the Ishikawa and the MCF-7 cell assays (for oestrogenic activity) and yeast cell assay (for oestrogenicity and androgenicity) are recommended. It is also necessary to assess the significance of sub-clones among the various cell lines. The potential use of transgenic models also requires investigation. Among the *in vitro* wildlife test models reviewed, none can be recommended for further development with the possible exception of the trout hepatocyte vitellogenin assay.

236. Structure-activity relationship models have been shown to be very useful as a preliminary screening tool during candidate selection of a highly characterised class of chemicals or in elucidating detailed mechanistic aspects. Clearly, available models are not sufficiently developed to permit their generalised use in the regulatory screening of novel chemicals, but their continued development is to be encouraged.

Table 5

Some non-regulatory test methods suggested for further development/validation

Test type	Model/species	Selectivity	Method	Endpoint(s)
Human-relevant – <i>in vivo</i>	Rat uterus	O	Weight of uterus in immature or ovariectomised rats	Uterine wet weight (possibly also mitotic rate, DNA content, etc.)
	Rat vagina	O	Assessment of induction of oestrus by smearing in immature or ovariectomised rats	Vaginal cornification assessed by vaginal smear/wash
	Rat prostate	A	Prostate weight in castrated rats	Prostate weight (possibly DNA/ RNA content or marker enzymes)
Human-relevant – <i>in vitro</i>	MCF-7 cells	O	Oestrogenic activity measured by increase in number of cells (Coulter counter), induction of oestrogen or progesterone receptors (using enzyme immunoassay kits) or exoprotein induction (gel electrophoresis); luciferase activity measured by luminescence	Oestrogen-dependent endpoints include: cell proliferation, levels of oestrogen or progesterone receptor or exoprotein; luciferase activity in transfected cells
	Ishikawa cells	O	Activity measured by increase in cell number or colourimetric assessment of alkaline phosphatase activity	Cell proliferation or alkaline phosphatase activity
	Transfected yeast	O/A	Oestrogenic/androgenic activity measured by simple colourimetric assay for reporter enzyme	Reporter gene activity, commonly β -galactosidase

A Androgens and/or anti-androgens

O Oestrogens and/or anti-oestrogens

Table 5 (continued)

Some non-regulatory test methods suggested for further development/validation

Test type	Model/species	Selectivity	Method	Endpoint(s)
Wildlife-relevant – <i>in vivo</i>	Vitellogenin assay	O	Possible to assess oestrogenic stimulation quantitatively using radioimmunoassay or ELISA kits for a wide range of oviparous species; may be possible to develop a (near-) universal kit; suitable for use in laboratory and field experiments	Vitellogenin concentration, principally in blood; also established for tissues and mucus
	Sexual dimorphism	O/A	Visual observation of changes in structure in response to sex hormone-disruption in fish	Changes in secondary sexual structures
	Multigeneration studies*	O/A	Assessment of reproductive success over several generations	Growth, behaviour, development, fecundity, hatching success/timing, etc.
Wildlife-relevant – <i>in vitro</i>	Hepatocyte culture #	O	Cell cultures of primary hepatocytes from male rainbow trout exposed to test chemicals at range of concentrations for two days; vitellogenin production assessed by radioimmunoassay	Vitellogenin concentration
Models	QSAR	O/A	Mathematical modelling of chemical interactions; requires additional development	Various

A Androgens and/or anti-androgens * Not a screening test
 O Oestrogens and/or anti-oestrogens # Possible, not definitive recommendation

5. FURTHER RESEARCH NEEDS

237. In the field of endocrine disruption there is a need to clarify the basic mechanisms of action (especially the importance of non-nuclear and nuclear receptor-mediated effects and of non-receptor mediated interactions). It is also necessary to rank the sensitivity of the various endpoints and to assess their relative importance as markers of toxicity. With regard to effects in wildlife species, there is a need to assess the ecological relevance of any selected endpoint. This process should include consideration of the relative benefit of incorporating additional endpoints into existing guidelines, compared with introducing a range of simple “stand alone” screens to detect specific endocrine-disruptive activities. Progress in the field is limited by the absence of chemicals accepted as suitable for use as reference materials. For some types of activity there is no general agreement as to which chemicals are active, whilst no chemical has yet been accepted as endocrinologically inactive (*i.e.* suitable for use as a negative control). Agreement on a set of reference chemicals would not only benefit basic scientific research in this area, but would also assist in method development and validation (particularly in respect of specificity and sensitivity and, where appropriate, in extrapolating from *in vitro* effect to the *in vivo* situation). An additional general concern for endocrine disruption is that the mechanisms may not follow a traditional dose-response pattern. Whilst it is well known in endocrinology that low concentrations can have opposite effects, this inverted-U shaped dose response relationship has recently been suggested for some endocrine-disrupting substances (vom Saal *et al.*, 1997). There is clearly a need to gain a better understanding of the dynamics of the responses, because this could have important consequences for the selection of appropriate dosages to study. The nature and likely frequency of occurrence of interactive effects resulting from exposure to combinations of endocrine-disrupting chemicals should also be the subject of further research and investigation. Moreover, it is necessary to assess whether the classical assumptions used during the regulatory process (*e.g.* that fish are predictive for amphibians and birds for reptiles, etc.) hold true for endocrine disruption. Finally, for the non-mammalian taxa there is a need to identify and characterise processes potentially susceptible to endocrine disruption that have no mammalian correlate, so that appropriate ecologically relevant test methods can be developed. This will involve the study of comparative endocrinology and physiology, with a view to identifying suitable representative species (especially among the invertebrates) for inclusion in an expanded testing strategy.

238. Focusing specifically on sex hormone-disruption, the mechanisms of action involving chemical-receptor interaction are the subject of on-going research. In particular, there is a need to clarify the significance of differences in receptor structure in different tissues and between species. Such differences in receptor type and function may have important implications for testing strategies to ensure adequate safeguards for humans and wildlife species. There is also a need to focus on possible alternative mechanisms of action, including interactions with the Ah-receptor, resulting in anti-oestrogenic activity via cross-talk at the level of the signal transduction pathways (*e.g.* for dioxins and polycyclic aromatic hydrocarbons), and alteration of the ratio of two major metabolites of oestradiol, the 2- and 16 α -hydroxyoestrones, in favour of the latter, which is a potent oestrogen and has been shown to have genotoxic properties. The development of assays to detect these and other mechanisms of action should be encouraged.

239. Many of the current screening tests use adult forms. However, there appear to be differences in sensitivity between adults and immature (*in utero* or *in ovo*) forms. Thus information is required on the predictability and significance of changes in the adult to the early life stage as this may be of profound

importance for risk assessment and could potentially necessitate development of alternative tests to assess effects during the early developmental stages.

240. To date, chemicals suggested as having oestrogenic or androgenic activity have been measured in humans as the chemical itself, a metabolite, or a specific marker effect. General biomarkers for overall exposure to oestrogens or androgens in humans do not yet exist. It would be useful to identify and develop such biomarkers for humans and other vertebrates, so as to assess total oestrogenic and androgenic burden, although this might prove difficult. It would also be useful to develop relevant biomarkers for a range of invertebrate species. In the case of wildlife monitoring, vitellogenin expression in males of oviparous species has been identified as a sensitive marker for oestrogenic exposure, although there are suggestions that other egg proteins such as the *zona radiata* proteins may be more sensitive. This situation requires clarification, whilst it would also be of assistance in laboratory and field work if a robust biomarker for androgenic modulation could be developed. In each of these cases, it is important to assess the relevance, not just the sensitivity, of the biomarker.

241. Current testing of chemicals is heavily focused on mammalian models because of their relevance to humans. However, it is known that there are processes in other taxa that do not occur in mammals but are potentially susceptible to endocrine disruption. To assist in the assessment of the potential ecological impact of new chemicals, and to enable meaningful ongoing monitoring programmes, it is therefore necessary to expand the scope of testing to include a wider range of taxa and endpoints. It may be appropriate to develop simple, inexpensive assays to span a range of species from a range of trophic levels (*e.g.* algae, molluscs, crustacea, insects and non-mammalian vertebrate groups) and, within levels if appropriate, a range of feeding strategies. Attention should be given to processes with no mammalian correlate (*e.g.* arthropod metamorphosis and moulting, sex ratio control mechanisms, etc.). Although they are probably not applicable as initial screens, there is also a need to extend the range of multigeneration study designs to facilitate investigation of subtle effects on reproductive effectiveness in a range of taxa. Possible additional models suggested at the European workshop referred to above (EC, 1997) that could be considered for development include those involving injection of chemicals either directly into eggs or via the mother in fish or bird species with the observation of subsequent developmental effects, and the development of transgenic fish with appropriate receptor and reporter systems that could then also be followed through to monitor general development. Further detailed recommendations were made for tests in mammals, birds and fish in the EMWAT report (Tattersfield *et al*, 1997).

242. The potential benefits of using structure-activity relationship models in the preliminary screening of specific classes of chemicals has been recognised. There is, however, a need for additional basic mechanistic research and continued development and refinement of these techniques before they can be considered for regulatory adoption.

243. Suggestions for future research activities are summarised in Table 10. Sex hormone and other endocrine disruptive processes are, however, a major focus of ongoing research activity. As new data are made available and knowledge of the underlying mechanisms increases, reassessment of the appropriateness of testing methods and risk assessment strategies will be necessary.

Table 6
Suggestions for future research activities

Area	Activity
Basic Science	Rank sensitivities of endpoints and assess their importance *
	Develop a reference set of chemicals of defined activity *
	Define dose-response profiles for endocrine-disruptive mechanisms/apply to dosage selection *
	Assess validity of classical toxicological assumptions of cross-group predictivity for endocrine-disruptive mechanisms *
	Clarify significance of differences in receptor structure in various tissues and species
	Undertake appropriate comparative endocrinology and physiology to support selection of suitable non-mammalian species for testing
	Elucidate non-receptor mediated effects
	Undertake additional mechanistic research and continued development and refinement of QSAR models
Screening model Development	Establish relative importance of <i>in utero</i> and <i>in ovo</i> exposure and apply this knowledge to testing strategies
	Develop test methods using a wide range of ecologically relevant (non-mammalian) test species *
	Develop novel approaches to screening <i>e.g.</i> transgenic fish
Biomarker research	Identify biomarkers of oestrogenic and androgenic responses in humans and wildlife
	Clarify whether vitellogenin or other egg proteins should be the marker of choice in oviparous species; assess their predictive value in terms of ecological importance

* Considered of high priority

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ANNEX

DETAILED BACKGROUND INFORMATION ON NON_REGULATORY TEST METHODS

INTRODUCTION

This section presents the detailed history, method and endpoints of those methods identified during critical review as being potentially suitable for adoption as new regulatory test methodologies. For convenience, the methods are presented in four major categories:

- *In vivo* systems relevant to assessment of potential toxicity to humans.
- *In vitro* systems relevant to assessment of potential toxicity to humans.
- *In vivo* systems relevant to assessment of effects on wildlife.
- *In vitro* systems relevant to assessment of effects on wildlife.

The critical assessment of these methods is in the Section Critical assesment of non-regulatory test methods.

IN VIVO SYSTEMS RELEVANT TO ASSESSMENT OF POTENTIAL TOXICITY TO HUMANS – OESTROGEN RELATED

RAT UTERINE MODELS

The rat models considered include one by O'Connor *et al.* (1996) which provided an integrated test battery for oestrogenic and dopamine-modifying chemicals. The endpoints used mainly related to uterine function or morphology; only those endpoints relating to oestrogen detection will be discussed in detail.

Bülbring and Burn (1935)

Bülbring and Burn (1935) reported on a comparison of bioassays for the estimation of oestrin and a male hormone present in oily solutions. This paper included details of a novel method of assessing the relative oestrogenic potency of solutions of unknown strength by comparison with the effects on uterine weight, following administration of known quantities of an oestrogenic hormone.

Method

In the initial experiment, Bülbring and Burn ovariectomised groups of three or four young female rats (approximately 40g). One litter mate was assigned to each group. Two days after ovariectomy, the animals were injected with varying dosages of oestrin. Dosing was repeated daily for the next three days, with the animals being humanely killed 48 hours after the last dose. Bodyweight was recorded and the uteri were dissected and placed in a modified Bouin's fluid. After 24 hours fixation, uteri were dried and weighed; data were expressed in terms of bodyweight-relative values and presented as a log dose versus weight graph.

Subsequently, using a similar experimental design, the strengths of solutions of oestrin were evaluated by blind comparison of uterotrophic activity against two groups receiving oestrin solutions of known strength. Initial ranging studies were performed to identify the concentrations necessary to cover those of the unknown solutions. The strengths of the unknown solutions were then derived from the graphical data presentations and recorded before breaking the blind.

Subsequent development

1. Uterine weight

Dorfman *et al.* (1936) investigated the oestrogenic activity of benzene-soluble, alkali-soluble and alkali-insoluble extracts of human male urine and extracts from bull testes using uterine hypertrophy and vaginal intotitus in rats. From day 25 of age, groups of five female albino rats received five daily injections of test material or known strengths of the oestrogens, theelin or theelol. Litter mates were assigned to each group. On day 30 the animals were killed and bodyweight and ovarian and uterine weights recorded. Reproducibility of results was assessed by repeating the theelin treatment on a further group of five rats.

Astwood (1938) reported on a method for the quantitative determination of oestrogen with an in-life phase of only six hours. This model was based on detailed experiments in which groups of rats were

humanely killed at varying times after subcutaneous injection with oestradiol and the uterine weight and fluid content assessed. A range of dosages were investigated in these studies. It was shown that there was no effect on uterine weight during the first hour, but a rapid (generally dosage-related) increase was seen thereafter which reached a maximum by six hours after dosing and subsequently declined. This early response was found to be largely attributable to changing water content. Subsequently, a further transitory increase in weight (not attributable to water content) occurred between 12 and 27 hours after dosing. Associated histopathology supported this finding. On the basis of these studies the following assay method was devised:

Three hundred animals received a series of dosages of oestradiol. A standard calibration curve was constructed for percentage increase in uterine weight (at six hours after dosing) against dosage. Dosages of 0.006 to 0.1 µg oestradiol were found to give a direct logarithmic relationship, and values were converted to standard units for convenience.

In order to assess the oestrogenic activity of an unknown solution, the author recommended its dilution in multiples of four and the injection of each dilute solution into groups of five animals derived from the same source as those used to construct the calibration curve. After six hours, the uterine weights are recorded and expressed as a percentage increase. The author anticipated that at least two of the dilutions used would fall within the range of the calibration curve and hence that the potency would be easily derived. For each experiment a control group, given either oestradiol or oestrone, was also included.

Hisaw (1959) reported on the relative potencies of 17β -oestradiol, oestrone, equilin, equilenin and diethylstilboestrol using a rat uterine fluid imbibition and growth model heavily influenced by the work of Astwood (1938).

Groups of 22-day-old Harvard female rats were given a single subcutaneous injection of one of the test materials in sesame oil, at a range of dosages. After four or six hours, the animals were killed. The uterus was dissected and weighed and then oven-dried at 100° to 115°C for 24 hours before determination of dry weight. For the initial experiments uterine nitrogen determinations were made, but these were discontinued because the data were found to closely parallel the absolute dry weight changes. In a number of cases the uterine weights were also determined following expression of luminal fluid. A four- and six-hour dose-response curve was derived for fluid imbibition. Data for uterine weight (wet, dry and as percentage water) were analysed for degree of uniformity within group, using standard error calculations. Other experiments performed by this author investigated the longer-term changes in uterine weight up to 70 hours following a single administration of an oestrogen.

Jones and Edgren (1973) compared the oestrogenic activities of a range of steroidal chemicals on the uterus and vagina of ovariectomised rats.

In this study, 30-day-old female rats were ovariectomised and, after a 12-day recovery period, received daily administrations of the test chemicals or vehicle alone for 14 days. Animals were necropsied 24 hours after the last dose and bodyweight, uterine and adrenal weight recorded. Organ weights were expressed as absolute (wet) weights. Samples of vagina were also taken for histopathological examination.

Wakeling and Bowler (1988) reported on a series of studies to investigate the potential anti-oestrogenic activity of a series of 7α -alkyl analogues of oestradiol. These experiments included the use of rat uterotrophic assays to distinguish between pure and partial antagonists.

Briefly, immature rats were dosed daily for three days with either the vehicle alone, oestradiol benzoate alone, or one of series of dosages of test material either alone or in combination with oestradiol benzoate. The uterine wet weights were recorded and expressed in bodyweight-relative terms. Agonistic and antagonistic activities were calculated using the following equations:

$$\begin{aligned}\% \text{ agonism} &= [(C-A)/(B-A)] \times 100 \\ \% \text{ antagonism} &= [(B-D)/(B-A)] \times 100\end{aligned}$$

where A, B, C and D are the bodyweight-relative uterine weights for vehicle alone, oestradiol alone, test material alone, and test material and oestradiol in combination.

In a further experiment, the effects of tamoxifen and what appeared to be a pure anti-oestrogen (ICI-164,384) were compared by dosing groups of intact female rats with either chemical daily for 14 days. Intact controls and ovariectomised animals were also included; these received daily injections of vehicle alone. The effects on uterine weights were compared.

Phillips *et al.* (1990) studied the pharmacological efficacy of norethindrone and ethynyl oestradiol using a range of laboratory models. This included an assessment of oestrogenicity using immature rats. Groups of 10 immature Wistar female rats received ethynyl oestradiol or norethindrone, either alone or in combination, daily by oral gavage for three days. On the fourth day of the study, the animals were killed and the uteri excised, trimmed and weighed. Data were analysed by Dunnett's test.

Ng *et al.* (1994) studied the hormonal activity of a natural plant product, yuehchukene, using a series of bioassays including a rat uterotrophic assay.

Sprague-Dawley rats were ovariectomised at day 18 of age. After a one-week recovery period, groups of five animals received test material or 17 α -ethynyl oestradiol alone or in combination by twice-daily oral gavage for three days. Twenty-four hours after the last dose, the uteri were removed and weighed with and without the uterine fluid contents. Data were expressed as absolute or bodyweight-relative values and as fluid- to dry-weight ratio (expressed as a percentage) and analysed by Student's t-test.

Dhar and Mattu (1995) reported on the use of immature female rats to investigate the oestrogenicity of o-hydroxynaphthaquinones.

Groups of five rats, weighing 40-50 g, were given three daily injections of either the vehicle alone, oestradiol valerate, the test material or the test material in combination with oestradiol. Twenty-four hours after the last dose, animals were killed and the uteri weighed.

A number of other studies have included uterine weight recording in addition to the use of biochemical markers. These include Bitman and Cecil (1970); Galand *et al.* (1987); Sheehan and Branham (1987); Astroff and Safe (1990) and Connor *et al.* (1996). These studies are considered in Section Uterine biochemistry receptor binding, below.

2. Uterine mitotic rate

During investigations into the oestrogenic activity of an alkylphenol (p-nonyl-phenol) released from polystyrene tubes, Soto *et al.* (1991) verified the effects seen in an MCF-7 human tumour line by use of a modification of the basic uterotrophic assay procedures, in which mitotic rate rather than weight was the endpoint.

Adult Sprague-Dawley female rats (120-150 g) were ovariectomised and primed with 15 ng of oestradiol on days 12, 13 and 14 after ovariectomy. On days 19 and 20, various dosages of the polystyrene-derived, purified alkylphenol were administered subcutaneously to the animals. Positive (1.25 µg oestradiol) and negative (vehicle alone) controls were included. Animals received intraperitoneal colchicine 20 hours after the last dose and were killed four hours later. Endometrial mitotic indices were assessed (method details not reported) and data statistically analysed using the Newman-Keuls test.

3. Uterine biochemistry/receptor binding

Although still based upon the uterotrophic response of the rodent to oestrogenic stimulation, a number of alternative endpoints to weight change have been developed.

Bitman and Cecil (1970) investigated the oestrogenic activity of DDT and 52 related chemicals using an 18-hour glycogen response test. The data derived were used to develop ideas on the relationship between chemical structure and biological activity. The test system involved the subcutaneous injection of test chemicals at a screening dose of 8 mg/rat to immature female Wistar rats. Animals were killed 18 hours after injection and the uteri weighed and analysed for glycogen content by an anthrone procedure. Substances identified as active were further tested at a range of dosages. Data were analysed by Student's t-test.

Galand *et al.* (1987) studied the short- and long-term effects of *o,p'*-DDT on the rat uterus using a series of endpoints and also tested the less oestrogenic derivative *p,p'*-DDT. Parameters monitored were uterine weight and RNA, protein, cyclic GMP and glycogen contents.

Female Wistar rats (21-22 days of age) received a single intraperitoneal injection of the test material. Positive (oestradiol) and negative (vehicle alone) controls were included. Five animals per group were killed after 3, 6, 18, 24, 48 or 72 hours. The effects of pre-treatment with the various DDT forms on uterine responsiveness to subsequent treatment with 17β-oestradiol were also investigated.

Sheehan and Branham (1987) reported on the potential influence of changing alpha foetoprotein levels with age on the *in vivo* oestrogenicity of three chemicals (oestradiol, diethylstilboestrol and ethynyl oestradiol) in rats. In addition to reporting on uterine weight response, a number of biochemical endpoints were also investigated to assist in clarification of early and late uterine responses. These included DNA and protein content and ornithine decarboxylase (ODC) activity.

CD rats were mated and the litter size reduced to seven or eight female pups within 24 hours of birth. Animals received daily subcutaneous injections of the appropriate oestrogen or vehicle alone for one of the following periods: days 1-5, 10-14, 20-24 or 60-64. Animals selected for dosing on days 60-64 were ovariectomised four or five days before the first injection to overcome the problem of cyclic oestrogen secretion in these mature animals. One hour after the last dose, animals were killed and the uteri weighed. A number of uteri were then oven-dried overnight to provide dry-weight data. Some animals from the day

5 and day 64 groups had their uterine cytosolic ODC activity measured six hours after the last treatment by assay of trapped $^{14}\text{CO}_2$ released from [^{14}C]ornithine.

Astroff and Safe (1990) investigated the anti-oestrogenic effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) using a rat uterus model stimulated by oestradiol treatment. The relative activities of 1,2,4,7,8-pentachlorodibenzo-p-dioxin (PeCDD) and 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) were also investigated as part of a structure-activity investigation. On the basis of previous work by a series of other authors, in which uterine peroxidase activity was shown to be an oestrogen-regulated enzyme, the authors selected this enzyme and organ weight as suitable endpoints for study.

In the dose-response study, female Sprague-Dawley rats (25 days of age) were given intraperitoneal injections of oestradiol alone or in combination with varying dosages of TCDD and killed after 48 hours. In the time course study, animals were similarly treated but also received further daily injections of oestradiol until termination after various times, up to 156 hours after the first dose. In the structure-activity investigation, animals received either TCDD, PeCDD or PeCDF alone or in combination with oestradiol and were killed 48 hours later. At humane killing, the uterine wet weights were recorded and expressed as bodyweight-relative values.

The uteri were homogenised, centrifuged at 39,000 g for 45 minutes at 2°C. The pellet was re-suspended in buffer, then similarly re-centrifuged. The final pellet was re-suspended in Tris-HCl and calcium chloride. Samples of this extract were added to a mixture of guaiacol and hydrogen peroxide and the initial rate (60 seconds) of guaiacol oxidation measured by spectrophotometry and expressed per gram of tissue. Data were subject to ANOVA.

Johri et al. (1991) used a uterine weight and peroxidase activity assay to assess the potential oestrogenic or antioestrogenic activity of clomiphene citrate, centchroman and embelin and of methanol extracts of the plants *Abutilon indicum* and *Butea monosperma*. Ovariectomised rats received oestradiol either alone or in combination with one of the test chemicals for three days, followed 24 hours later by humane killing and weighing of the uterus. The uteri were then homogenised, centrifuged, and the supernatant assessed for peroxidase activity using a spectrophotometric assay.

Bigby and Young (1994) studied the oestrogenic effects of the antiprogestin onapristone, using a series of rodent *in vivo* and *in vitro* models. These included measurement of DNA synthesis and histomorphometric examination of the rat uterus.

Sprague-Dawley rats (21 days old) were given the test material by subcutaneous injection. Twenty hours later the rats were injected with tritiated thymidine before necropsy. Uterine specimens were formalin-fixed, paraffin-fixed, and sections autoradiographed to determine the thymidine labelling index. Epithelial cell height and stromal cell density (as a measure of stromal oedema) were determined using a morphometric programme and a microscope equipped with a drawing tube and digitising pad. A minimum of 100 epithelial cells were measured per animal, and 2000 to 3000 cells assessed for the stromal cell density measurement. Statistical analysis was by t-test or analysis of variance, followed by Fisher's F-test.

Tennant et al. (1994) studied the interactions between the triazine herbicides atrazine, and simazine and a metabolite, diaminochlorotriazine, and oestrogenic activity using various uterine endpoints in rats. Animals were dosed with the test chemicals and/or oestradiol. Uterine weight effects were studied in young-adult ovariectomised animals using a three-day treatment period and group sizes of six to seven animals. Animals were humanely killed 24 hours after the last dose and absolute uterine weight was recorded. Progesterone receptor levels were also assessed in groups of five to six, and ovariectomised rats

treated for two days. Uteri were homogenised and co-incubated with tritiated R-5020 to derive the specific binding. Thymidine incorporation was assessed in sexually immature animals using groups of six to seven animals treated for two days, followed by humane killing 24 hours after the last dose. Samples of the uteri were homogenised and co-incubated with radiolabelled thymidine, followed by incorporation measurements using liquid scintillation methods.

Connor *et al.* (1996) studied the mode of action of two chloro-s-triazine-derived compounds, atrazine and simazine, using a range of models. These included the measurement of uterine progesterone receptor binding activity, weight, and peroxidase activity.

Female Sprague-Dawley rats (21 days of age) were assigned to treatment groups as follows (group size was four for the treated groups and five in the controls):

Group	Treatment	Group	Treatment
1	Control	9	Control
2	10 µg/kg/day - oestradiol	10	10 µg/kg/day - oestradiol
3	50 mg/kg/day - atrazine	11	10 µg/kg/day - oestradiol +50 mg/kg/day - atrazine
4	150 mg/kg/day - atrazine	12	10 µg/kg/day - oestradiol + 150 mg/kg/day - atrazine
5	300 mg/kg/day - atrazine	13	10 µg/kg/day - oestradiol + 300 mg/kg/day - atrazine
6	50 mg/kg/day - simazine	14	10 µg/kg/day - oestradiol + 50 mg/kg/day - simazine
7	150 mg/kg/day - simazine	15	10 µg/kg/day - oestradiol + 150 mg/kg/day - simazine
8	300 mg/kg/day - simazine	16	10 µg/kg/day - oestradiol + 300 mg/kg/day - simazine

Animals received the appropriate treatment by daily oral gavage for three days. They were killed 20 hours after the last treatment, and uteri were weighed and bisected. Uterine bisections were assigned to progesterone receptor (see below) or peroxidase assays.

For the peroxidase assay, uterine bisections were pooled and homogenised. Homogenates were centrifuged at 39,000 g at 2°C for 45 minutes. After washing, the pellet was resuspended in Tris buffer and the suspension clarified by re-centrifugation. Peroxidase analysis was then undertaken using a procedure similar to that detailed above for Astroff and Safe (1990).

Uterine horn bisections were pooled for each group in ice-cold TESHMo, multiply homogenised, centrifuged at 105,000 g for 45 minutes, and the supernatant (cytosol) decanted for immediate receptor binding assay. After incubation with [³H]R5020 in the absence or presence of 2 µm unlabelled progesterone for 16 to 18 hours, the cytosol was stripped using dextran-coated charcoal. After further centrifugation (5000 g for 10 minutes), the radioactivity of the supernatant was measured by liquid scintillation counter. Receptor levels were reported in fmol/uterus, assuming a stoichiometry of one R5020 molecule bound per receptor. Three determinations were performed per pooled extract.

3. *In vivo* test battery (O'Connor *et al.*, 1996)

O'Connor *et al.* (1996) reported on the use of an integrated test battery based upon the ovariectomised rat. This included various endpoints, the majority of which related to various aspects of uterine function. Chemicals examined included the oestrogens oestradiol and oestriol, the mixed-function oestrogen agonists/antagonists, tamoxifen, a full antioestrogen, ICI-182,780, and the dopamine modulators haloperidol and reserpine.

Female CD rats were ovariectomised at 41 days of age and maintained under standard conditions. One week later, the animals were randomly assigned to groups on the basis of bodyweight. Fourteen animals per group were assigned to hormonal or biochemical investigations and six per group were used for cell proliferation/morphometric evaluation. These latter six animals per group were surgically implanted with Alzet osmotic pumps containing 5-bromo-2'-deoxyuridine in sodium bicarbonate buffer on the day before treatment commenced. Test materials were administered by intraperitoneal injection three times each day for four days. Bodyweight was recorded daily during the course of the study. Rats assigned to hormonal or biochemical investigations were evaluated for vaginal cytology by the collection of daily vaginal washes and preparation of slides. These were stained using the Wright-Giemsa method and evaluated for conversion out of diestrus.

On test day 5, animals were killed by exsanguination within a three-hour period. Blood was collected from all animals and the serum stored deep-frozen. This was assayed for prolactin levels as a marker of oestrogenicity, using a commercial radiimmuno assay kit. The presence of fluid in the uterine horns was recorded, and uteri scheduled for hormonal or biochemical investigations were weighed and processed to the cytosol stage. These uteri were pooled two per tube, mechanically homogenised in a glycerol/Hepes/EDTA/dithiothreitol/sodium molybdate/sodium fluoride/pefabloc/ leupeptin buffer. After centrifugation at 68,000 g for 90 minutes, the supernatant was deep-frozen pending hormone receptor assay. Oestrogen and progesterone receptor concentrations were measured using a single saturating concentration of the appropriate radiolabelled ligand. Non-specific binding was determined using a 250-fold excess of non-labelled competitor (diethylstilboestrol for oestrogen and promegestone for the progesterone receptor).

Uteri from animals scheduled for cell proliferation/morphometric analysis were weighed, fixed to dental wax, and placed in Bouin's solution for two hours. This was followed by routine histological processing to produce sections at 5µm. Uterine sections were stained for immunohistochemical analysis of bromodeoxyuridine incorporation into DNA, using an avidin-biotin-peroxidase complex method employing a monoclonal antibody to bromodeoxyuridine and DNA substrate. Labelling indices for uterine endometrial stromal cells were determined for 1000 cells. Additional sections were prepared for morphometric analysis from the uteri of these animals. Four or five transverse sections were prepared from the proximal end of the uterus at 2-3 mm intervals. These were embedded proximal end down, and a consistent pattern of trimming and embedding was used to identify individual sections proximal to distally along the uterus. Epithelial cell height was measured on haematoxylin and eosin-stained sections using an image analyser. For each animal, uterine epithelial height was taken from the average measurement of a 350 µm basement membrane from each of three uteri cross sections. Routinely the second, third and fourth sections were used. Uterine stumps were also examined histopathologically to confirm the absence of ovarian tissue.

Bodyweight, organ weight, cell proliferation, uterine morphometry measurements, and hormone and receptor levels were analysed by one-way ANOVA. When the corresponding F-test was significant, pairwise comparisons were made by Dunnett's test. Bartlett's test for homogeneity of variances was performed, followed, when significant, by non-parametric analyses.

In a subsequent experiment, animals were subject to dietary restriction in order to determine if any of the endpoints showed bodyweight dependency. Dietary restriction levels used for this study were *ad libitum* or 17, 14, 11 or 8 g/day/animal. These were based upon the calculated food intake data from previous work.

Endpoints/data interpretation

Bülbring and Burn (1935); Dorfman *et al.* (1936); Astwood (1938); Hisaw (1959); Jones and Edgren (1973); Wakeling and Bowler (1988); Phillips *et al.* (1990); Ng *et al.* (1994); Dhar and Mattu (1995): Uterine weight: expressed as:

- wet absolute and bodyweight-relative values and as % fluid to dry weight (Ng *et al.*, 1994).
- bodyweight-relative values for fixed tissue (Bülbring and Burn, 1935);
- absolute and bodyweight-relative (less gut) weights for fresh tissue (Bülbring and Burn, 1935);
- percentage increase for wet tissue, expressed in Astwood units¹ (Astwood, 1938);
- wet and dry absolute values and as percentage water (Hisaw, 1959);
- bodyweight-relative wet weight (Wakeling and Bowler, 1988; Dhar and Mattu, 1995); and
- absolute wet weight (Jones and Edgren, 1973; Phillips *et al.*, 1990).

¹ amount of oestrogen which, injected subcutaneously in 0.1 c.c. sesame oil into 21 to 23-day-old rats, produces on average a 34% increase in uterine weight after six hours.

In the paper by Bülbring and Burn (1935), an acceptable level of accuracy from the test methodology was reported and it was observed that it was possible to assess the strength of an unknown solution within two weeks using this method, as compared with three weeks for a vaginal smear method. Dorfman *et al.* (1936) found oestrogenic activity in human urine extracts and showed differential activities for known oestrogens with respect to vaginal and uterine endpoints. They also reported good reproducibility of results and showed that there was little difference in the significance of the results when absolute or bodyweight-relative uterine weights were considered. The method of Astwood (1938), although requiring extensive initial development of a calibration curve, permitted rapid assessment of activity for uncharacterised materials and expressed results in a standardised form. In further studies on a range of oestrogens, Hisaw (1959) demonstrated clear effects on the uterus in terms of wet, dry, and percentage water. The oestrogens differed in their ability to promote fluid imbibition; oestradiol was selected as the standard for comparison. The maximal response time varied depending upon dosage, with higher dosages tended to shorten the time to maximal response. At the highest level the initial effect was seen to be depressed compared with that at lower dosages. The longer-term changes in uterine weight were not attributed to fluid imbibition, but were considered to represent true tissue growth. Of importance was the author's finding that the effectiveness of the various oestrogens at initiating fluid imbibition and uterine growth were independent, *i.e.* the relative potencies for each endpoint were markedly different. In the paper by Jones and Edgren (1973), a wide range of steroids were also shown to cause an increase in uterine weight, although in some cases it was necessary to use high dosages to elicit a response. This study, which also investigated the vaginal histopathological responses elicited by these chemicals, is reviewed further in Section 0.

Wakeling and Bowler (1988) demonstrated that a modified test procedure using immature animals permitted the quantitation of relative agonistic and antagonistic activities for a series of chemicals by comparing their effects either alone or when co-administered with oestradiol. In further studies they investigated the longer-term effects of one of these chemicals (which they had identified as a pure

antagonist) on intact mature rats using uterine weight as the endpoint. Results were expressed as percentage of maximum reduction in weight achieved by ovariectomy; tamoxifen was found to cause a maximum of 60% reduction compared to that seen on ovariectomy, whilst ICI-164,384 resulted in a 90% effect. Phillips *et al.* (1990) demonstrated a dosage-related response in the weight effect following ethynyl oestradiol treatment at 2-32 µg/kg (maximal response). Administration of norethindrone alone showed a potency of only 0.002 times that of ethynyl oestradiol, whilst administration in combination with the oestrogen resulted in no significant effect on ethynyl oestradiol activity. Ng *et al.* (1994) were able to demonstrate that, on the basis of uterine weight changes, yuehchukene had oestrogenic agonist activity when given either alone or in combination with 17 α -ethynyl oestradiol, although the response was much attenuated compared to what could be achieved when 17 α -ethynyl oestradiol was given alone. Dhar and Mattu (1995) reported that oestradiol valerate induced a marked increase in uterine weight after three days of treatment, whereas the test materials did not. When given in combination, a reduction in effect relative to treatment with oestradiol alone was seen.

Uterine mitotic rate

Soto *et al.* reported that the endometrial mitotic index induced by 50 mg of alkylphenol was significantly higher than that for ovariectomised animals but lower than for oestradiol-treated animals. Evidence of dosage relationship was recorded, with 20 mg showing only a weak positive response whilst 10 mg of the alkylphenol gave values similar to the negative controls.

- **Uterine weight:** assessed as absolute wet weight.
- **Uterine glycogen:** in terms of µg/100 mg dry weight and µg/uterus.

Bitman and Cecil (1970): A range of activities for the chemicals was reported. Potency was assessed in terms of the minimal dose that would increase glycogen to a level significantly different from the control values. The results obtained were compared with the data from uterine weight recording; the response line was steeper for the glycogen effect than for uterine weight. On the basis of these studies, the authors considered that oestrogenic activity in the chemicals examined was dependent upon the *p* or *p'* position being unoccupied (-H) or substituted with -OH or -OCH₃ groups and that a stable ethane chain was necessary. Halide or alkyl groups in these positions were found to render the chemicals oestrogenically inactive.

Galand *et al.* (1987):

- **Uterine weight:** expressed as percent of negative control value.
- **Uterine RNA, protein, cyclicGMP and glycogen contents:** expressed as percentage of negative control value.

o,p'-DDT and 17 β -oestradiol treatment elicited transitory changes in wet weight and RNA, protein and glycogen contents with high levels at 24 hours declining to near control levels by 48 hours. Levels changed more slowly over the first 18 hours for *o,p'*-DDT, whilst effects for *p,p'*-DDT-treatment were much less. The changes in uterine cGMP were also similar for *o,p'*-DDT and 17 β -oestradiol-treated animals at three and six hours, with no significant difference from the negative control being seen for the

p,p'-DDT form. Effects of oestradiol treatment following pre-treatment with DDT showed a maintenance of responsiveness.

Sheehan and Branham (1987):

- **Uterine weight:** expressed as bodyweight-relative values.
- **Uterine DNA and protein content:** expressed as µg/uterus/10 g bodyweight.
- **Uterine ornithine decarboxylase activity:** expressed as nmoles CO₂/mg protein/hour.

It was necessary to adjust for bodyweight in order to account for the large differences in uterine weight across the different age groups, and because of individual differences in weight between animals of the same age group. At each age tested, dosage-dependent increases in uterine wet weight were seen over about four logs of dosage. Within each age group, the slopes of the linear portion of the dose-response curves for the three hormones were not significantly different when tested for parallelism. A similar response was observed for the dry weight data. Measurement of uterine DNA and protein levels for oestradiol-treated neonatal animals showed a similar dose dependency as for uterine weight. A comparison of wet weights (calculated as a percentage of maximal response) showed alterations in potency with age, with the young animals showing least response although there were differences for each oestrogen. In contrast, measurement of ODC activity, demonstrated that the oestrogens would uniformly induce a six-hour peak in activity at all the ages examined, although an 18-hour peak was also observed in the older animals. The authors considered that the different profile for ODC activity, when compared with other endpoints examined, might result from different mechanisms at the level of hormonal entry into the cells or subsequent intracellular events.

Astroff and Safe (1990); Connor *et al.* (1996):

- **Uterine weight:** expressed as bodyweight-relative (Astroff and Safe) or absolute (Connor *et al.*) wet weights.
- **Uterine peroxidase activity:** by spectrophotometry.
- **Uterine progesterone receptor levels:** by liquid scintillation count; reported as fmol/uterus (Connor *et al.* only).

Astroff and Safe noted that the peroxidase activity peaked after 24 hours for the oestradiol-treated animals and remained elevated for the entire study period. Co-administration of TCDD or PeCDF resulted in a significant antagonistic effect, which was clearly apparent from 24 hours after dosing; corresponding effects were seen for uterine wet weights. PeCDF was, however, inactive as a uterine anti-oestrogen.

Connor *et al.* demonstrated that oestradiol induced all three endpoints examined; co-treatment with atrazine and simazine did not influence weight gain, but its manner of inhibiting the peroxidase activity and receptor-binding levels was not dosage-related. In addition, atrazine and simazine treatment alone decreased uterine receptor levels and peroxidase activity; uterine weight was not affected. The authors concluded from these and other studies that the mechanisms responsible for eliciting the inhibitory effects that were not dose-dependent were separate from the oestrogen receptor.

Johri *et al.* (1991):

Uterine weight: expressed relative to bodyweight.

Uterine peroxidase activity: expressed as change in absorbance/mg protein/minute.

Clear dose-response was shown following oestradiol treatment for each endpoint, but the percentage of difference was more marked for the peroxidase endpoint. Clomiphene citrate, centchroman and *Abutilon indicum* extract inhibited the effect of oestradiol in a dose-dependent manner, but showed a stimulatory effect when given alone. In contrast, embelin and *Butea monosperma* extract were stimulatory when administered alone or in combination with oestradiol.

Biggsby and Young (1994):

Uterine DNA synthesis: assessed by autoradiography.

Uterine epithelial hypertrophy and stromal oedema: by histomorphometric examination.

Increased uterine epithelial DNA synthesis following onapristone treatment was reported, together with an increase in cell height and stromal oedema.

Tennant *et al.* (1994):

Uterine weight: expressed as absolute values.

Uterine progesterone receptor-binding capacity: by derivation of specific binding capacity; expressed as binding capacity per mg cytosol protein.

Uterine DNA synthesis: established by measurement of thymidine incorporation; expressed as cpm radioactivity per µg DNA.

Treatment with oestradiol, in conjunction with triazine herbicide treatment at 300 mg/kg, resulted in lower uterine weights than when animals received oestradiol alone. At this dosage, in the absence of oestradiol, no effects on thymidine incorporation were seen; however, effects were detected at 50 mg/kg or above when given in combination with oestradiol treatment. Effects on progesterone receptor-binding were only apparent at 300 mg/kg in conjunction with oestradiol treatment. The authors interpreted their findings as indicating that these chemicals are not inherently oestrogenic but are weak oestrogen antagonists.

O'Connor *et al.* (1996):

Serum prolactin levels: using a commercial radioimmuno assay.

Uterine weight: expressed as absolute values.

Uterine fluid levels: by visual observation; scored as presence/absence.

Uterine epithelial cell height: by morphometric analysis using an image analyser.

Uterine stromal cell proliferation: by immunohistochemical analysis of bromodeoxyuridine incorporation into DNA.

Uterine progesterone and oestrogen receptor numbers: by radioassay.

Vaginal cornification: conversion out of diestrus assessed by daily vaginal washing/slide examination.

A dosage-related decrease in final bodyweight for all treatments was recorded, except for ICI-182,780. In some cases the decreases were marked, potentially resulting in a confounding effect upon the various endpoints measured. In the food restriction study subsequently conducted by the authors, bodyweight restriction of up to 80% was attempted. Of the endpoints considered for oestrogenicity, only uterine progesterone receptor numbers were considered to show bodyweight dependency.

A number of effects of treatment with the various chemicals were observed. The presence of fluid in the uterus was only seen in animals given oestradiol or oestriol, whilst these chemicals and the mixed agonist/antagonist tamoxifen affected oestrus conversion and uterine stromal cell proliferation and increased absolute uterine weight values. These chemicals did, however, result in different levels of response between the various endpoints. Epithelial cell height was found to be increased by these chemicals, but the dopamine antagonists haloperidol and reserpine also initiated a similar, but less marked, effect for this endpoint. Uterine oestrogen receptor concentrations were decreased in a dose-dependent manner for all test compounds, the effects being statistically significant for all except haloperidol. In contrast, uterine progesterone receptor content was statistically increased in rats treated with oestradiol, oestriol or tamoxifen but significantly decreased in the case of haloperidol, reserpine and ICI-182,780. The effects of oestradiol and oestriol were not dosage-dependant. In the case of haloperidol and reserpine, the effects were attributed to the influence of reduced bodyweight. However, the effect of ICI-182,780 was considered to represent a true effect of treatment. Serum prolactin levels were significantly increased statistically in animals given oestradiol, tamoxifen, or either of the dopamine modulators.

In reviewing the performance of this test battery, the authors commented that uterine weight was only minimally affected by weak oestrogens, thus making definitive conclusions as to the oestrogenicity difficult. However, they considered that under the experimental conditions of this model it was possible to distinguish between full and weak oestrogenic agonists by the magnitude of uterine weight increase. Vaginal cornification (oestrus conversion) permitted sampling without the need to kill the animal and was a very specific response to oestrogenic stimulation. This endpoint did present problems with characterisation of dose-response. Other endpoints, such as uterine fluid imbibition and stromal cell proliferation, were also considered valuable tools. Uterine fluid imbibition was a very specific but not very sensitive endpoint, because other classes of chemicals such as progestagens and testosterone can exert a similar effect at high levels.

O'Connor *et al.* noted, on the basis of the dose-response curves obtained in these experiments, that uterine stromal cell proliferation was the most sensitive and specific endpoint for oestrogenic stimulation. In contrast, the greatest effect on uterine cell height was made by the mixed agonist/antagonist tamoxifen, followed by the full oestrogen agonist oestradiol and the partial agonist oestriol. This might reflect the dosages used or differences in relative binding affinity for the oestrogen receptor. The dopamine modulators also affected this parameter. This could arise from the stimulatory effect of these compounds on serum prolactin levels. Hence, it was concluded that uterine epithelial cell height is a sensitive but not specific marker of oestrogenic compounds. Measurement of uterine progesterone and oestrogen receptor levels may be of value, but these endpoints require cautious interpretation due to the lack of specificity and, in the case of progesterone receptor levels, of bodyweight dependency. Serum prolactin appeared to respond to exposure to both oestrogen and dopamine modulators, so that any change in this parameter would need to be interpreted in the light of changes in other endpoints. In conclusion, the authors

remarked the unique profile of effects found with oestrogen receptor agonists and suggested that full and partial agonists could be distinguished on the basis of the magnitude of the responses seen. Measurement of prolactin levels should permit the classification of dopamine modulators from oestrogenic agents, whilst it should be possible to distinguish oestrogen antagonists from agonists on the basis of their effects on oestrogen receptors, progesterone receptors and uterotrophic responses.

RAT VAGINAL MODELS

Allen and Doisy (1924) investigated the effect of a porcine-derived, ovarian follicular extract (*i.e.* having oestrogenic activity) on the growth and functional development of the reproductive tract of immature female rats. This paper served to establish two important markers of oestrogenic stimulation, namely vaginal opening and induction of oestrus.

Method

In a series of experiments, immature rats (24-54 days of age) received injections of either vehicle alone, the liquor folliculli from large pig ovarian follicles, or an alcohol-soluble extract from the follicles. (Dosages were expressed in terms of "rat units", one rat unit being was the minimal quantity required to induce full oestrus growth in the genital tract of a spayed adult rat 48 hours after the first of three 1 cc injections, given at intervals of four to six hours.) Animals from each litter were assigned to treated and control groups and the effects of various numbers of administrations were investigated. Both intact and ovariectomised animals were investigated.

Animals were monitored for vaginal opening, and vaginal smears were prepared and examined for oestrus. In addition, the vagina and uterus were examined histopathologically from representative control and treated animals.

Subsequent development

Vaginal opening/smearing

BÜlbring and Burn (1935) used the monitoring of vaginal smears to establish the occurrence of oestrus in ovariectomised rats treated with a range of concentrations of oestrin in oily solutions.

Dorfman *et al.* (1936) similarly monitored vaginal opening as a marker of oestrogenic stimulation, as part of their experiments on extracts of human urine and bull testes. In these studies, groups of three to five juvenile albino rats (25 days of age) received five daily injections of the test material or control. Litter mates were assigned to each group, and the animals were examined daily for vaginal opening throughout the treatment period. Incidences of vaginal opening were expressed as a percentage of animals in the group showing opening.

Deckers and Schuurs (1989) used a modification of the original methodology of Allen and Doisy to determine the oestrogenic activity of a range of oestrogen precursors used during investigations into aromatase inhibitors (see Section 0). Female rats weighing 150-200 g were ovariectomised and, after a three-week recovery period, were primed with a single injection of oestradiol. Seven days later, the test chemicals were administered daily for four days. Chemicals studied included 19-mercapto-androstenedione, 4-hydroxyandrostene-dione and 19-methylene-androstenedione. Anti-oestrogenicity was similarly assessed by co-administration of oestradiol and the test material to ovariectomised rats for 10 days. Vaginal smears were obtained and the number of positives recorded.

Phillips *et al.* (1990) included the study of rodent vaginal cornification responses in their detailed investigations into the pharmacology of norethindrone and ethynyl oestradiol. Ovariectomised adult females were treated, at least seven days after surgery, by oral gavage administration for two days with either of the test chemicals. Vaginal washings were examined on days 3 and 4 of the study, with a positive oestrogenic response being assigned where cornified cells were detected in any of the washings. The dose stimulating cornification in 50% of the animals was calculated.

The anti-oestrogenic potential of norethindrone was also assessed by measuring its ability to inhibit oestrogen-induced cornification. In this experiment, ovariectomised animals received co-administration of norethindrone and 17β -oestradiol for four days. Vaginal washings were taken on the day following the last treatment. Absence of cornified cells indicated inhibition of the oestrogen response. The dose inhibiting oestrogen-induced cornification in 50% of the animals was also calculated.

Vaginal morphology

Jones and Edgren (1973) studied the effects of various steroids upon the vaginal histology of the ovariectomised rat. Chemicals investigated included ethynyl oestradiol, mestranol, methyl testosterone, hydrocortisone, progesterone, lynestrenol, norethisterone, norethisterone acetate, norethyndrel, norgestrel and ethynodiol diacetate. A comparison was also made of the uterine weight responses seen.

Thirty-day-old female rats were ovariectomised and, after a 12-day recovery period, received daily administration of the appropriate test chemical or vehicle alone for 14 days. Animals were necropsied 24 hours after the last dose and bodyweight, uterine and adrenal weights recorded. Organ weights were expressed as absolute (wet) weights. Samples of vagina were also taken, fixed, stained with haematoxylin and eosin, and histopathologically examined.

Vaginal morphology and biochemistry

Kronenberg and Clark (1985) used a range of biochemical assays to monitor keratin production in the rat vagina in response to treatment with a range of chemical types. The chemicals were comprised oestradiol, oestriol, diethylstilboestrol, and the mixed agonist-antagonist chemicals enclomiphene and zuclomiphene. The results of associated histopathological examinations were used to assist in the interpretation and direction of the biochemical investigations.

Mature female Sprague-Dawley rats were ovariectomised. After a seven- to 10-day recovery period, animals received the appropriate test chemicals at a range of dosages and during various treatment periods. Animals were killed at the end of the relevant treatment period and the vagina excised for further study. A number of investigations were performed during the course of a series of experiments.

After 24-hour fixation in Bouin's fluid, samples were processed and sections stained with Masson's Trichrome before light microscopic examination. For other endpoints investigated, fresh vaginal epithelial cells and sheets were isolated and extracted in buffer. DNA content analysis was achieved by centrifugation, drying, sonification and incubation with perchloric acid solution for 30 minutes. The precipitate was then analysed by the Burton method. In addition, protein levels were determined using the method of Bradford. Extracts were also polyacrylamide gel electrophoresed with densitometry measurement by optical scanning to identify protein subcomponents. Changes in the individual protein subunits in the vaginal extracts were monitored over time using immunoblotting and immunodotting techniques. Epithelial proteins were separated by polyacrylamide gel electrophoresis, transferred to nitrocellulose, and exposed to keratin antisera. Antibody binding was monitored by [125 I]protein A and visualised by autoradiography using dried mounted blots stored at -20°C .

ENDPOINTS/DATA INTERPRETATION

Allen and Doisy (1924); Bülbring and Burn (1935); Dorfman *et al.* (1936); Jones and Edgren (1973); Deckers and Schuurs (1989):

- **Vaginal opening:** by visual observation (Allen and Doisy, 1924; Dorfman *et al.*, 1936).

- **Induction of oestrus:** assessed by examination of vaginal smears (Allen and Doisy, 1924; Bülbring and Burn, 1935; Deckers and Schuurs, 1989).
- **Vaginal and uterine morphology:** by light microscopic examination (Allen and Doisy, 1924; Jones and Edgren, 1973).

Allen and Doisy (1924) found that treatment of immature rats with ovarian follicular material resulted in the induction of a sexually mature condition similar to that normally experienced by an animal at its first oestrus. A positive response could be elicited in animals as young as 26 days of age, within two or three days following receipt of four to six treatments of the hormonally stimulatory chemicals.

Bülbring and Burn (1935) assessed the induction of oestrus by examination of vaginal smears. The authors used this established endpoint as a comparator against the uterine weight method they had developed using ovariectomised rats. Similarly, Dorfman *et al.* (1936) used vaginal opening as an early indicator of oestrogenic stimulation in their studies on human urine and bull testis extracts and effects on uterine weight. The details of these experiments are further reviewed in Section 0. The experiments of Deckers and Schuurs (1989) demonstrated no oestrogenic activity for the chemicals studied, but there was a suggestion of an anti-oestrogenic effect at high dosages of 4-hydroxyandrostene-dione and 19-methyleneandrostenedione.

Jones and Edgren (1973) used formation of a well defined keratinised epithelial layer to define a positive vaginal response; results were expressed as an estimated ED₅₀ and subsequently expressed as a percentage relative to ethynyl oestradiol. The majority of chemicals tested were positive at one or more dosages, with ethynyl oestradiol being the most potent and norethisterone requiring the largest dose to produce a positive response. Vaginas of the vehicle control group showed an atrophic epithelium only two to three cells in thickness. The vagina of those receiving hydrocortisone, methyltestosterone, progesterone or norgestrel were indistinguishable from the controls. The authors commented on the differences in response seen for the vaginal and uterine endpoints, with positive uterine responses being recorded for all compounds although very high dosages were required in some cases. For ethynyl oestradiol and mestrandol, the results obtained from uterine organ weight analysis qualitatively reflected those based upon the vaginal histology, whilst the uterine responsiveness seen for hydrocortisone, methyltestosterone, progesterone or norgestrel was considered to reflect the lower specificity of this endpoint compared to vaginal keratinisation.

Phillips *et al.* (1990):

- **Induction of oestrus:** assessed by examination of vaginal washings for the presence of cornified cells.

Ethynyl oestradiol induced vaginal cornification in a dosage-related manner. Very high dosages of norethindrone (over 100 times more than for ethynyl oestradiol) were necessary to elicit a response, although at the dosages examined an anti-oestrogenic response was not detected when co-administered with oestradiol.

Kronenberg and Clark (1985):

- **Vaginal morphology:** by light microscopic examination.
- **Vaginal DNA content:** determined by the method of Burton.
- **Vaginal protein content:** determined by the Bradford method.
- **Vaginal keratin changes:** using immunoblotting and immunodotting techniques.

Single doses of oestradiol or diethylstilboestrol produced similar protein patterns, as assessed by SDS gels. Enclomiphene showed a diminished pattern compared with that for oestradiol, whilst zuclomiphene showed greater agonistic activity, producing a fainter but full pattern. Multiple dosages of oestradiol and oestriol produced a more marked pattern. At no stage were the responses to enclomiphene or zuclomiphene as great as that to oestradiol. Associated light microscopy on samples taken from animals given a single treatment of oestradiol or diethylstilboestrol showed a staged development of the vaginal epithelium, with full development occurring after 48 hours. Two treatments with oestriol evoked a response similar to that seen 24 hours after a single dose with oestradiol, whilst similar responses were seen with the other chemicals. A marked increase in the protein fractions representing keratin occurred following treatment; a close association with the time course and extent of tissue growth was also noted. Six major protein bands were identified as markers of oestrogen activity, although it was found that there were stage-specific increases for each band. Characteristic patterns were established for each time point for oestradiol treatment, and these data were then used as a reference to evaluate the oestrogenic potential of the other chemicals quantitatively.

Because the results of the histopathology and gel densitometry investigations were in close agreement, the authors progressed to the immunological methods to permit more sensitive measurement of keratin levels; the 53K antiserum was chosen because of its well defined specificity. Immunoreactive keratin concentrations clearly followed the patterns established in the gel densitometry studies.

Overall, it was found that keratin levels accurately reflect the observed changes in tissue maturation and can serve as measures of agonist properties. When they were normalised by expressing on a "per μg DNA basis" (to compensate for differences in synthesis and recovery), it was possible to differentiate between the effects caused by oestradiol and diethylstilboestrol. The latter chemical showed a 25-60% higher activity, which it was suggested might reflect a difference in metabolism. All the chemicals investigated appeared to have a common site of action, and this might indicate that the activities of agonists and antagonists may be exerted at the same genetic site.

ANDROGEN-RELATED

RAT PROSTATE MODELS

There is a considerable body of literature available to document the involvement of sex hormones in the development and functioning of the rodent prostate. In Section Mouse Prostate Models, variations of a model using the mouse prostate to assess oestrogenic effects of chemicals are reviewed. A number of workers have developed methods designed to utilise the sensitivity of the rat prostate to androgenic stimulation or anti-androgenic agents. These are reviewed below.

In vivo androgen receptor affinities

As part of a series of experiments investigating the ability of progestins to antagonise the biological actions of androgens (*i.e.* to behave as anti-androgens), Botella *et al.* (1987) investigated changes in the cytosolic and nuclear androgen receptors following *in vivo* exposure of the rat prostate to chemicals. Chemicals investigated included mibolerone, medroxyprogesterone acetate, nomegestrol acetate, megestrol acetate, chlormadinone acetate and cyproterone acetate.

Method

The study was performed as five replicates. For each replicate, a group comprised two adult male rats. Animals were castrated exactly 24 hours before intraperitoneal injection with test material or vehicle alone. One hour after administration, the prostates were removed. The prostate tissue was centrifuged at 800 g for 10 minutes and the supernatant separated. This was further centrifuged at 105,000 g for one hour to yield a cytosol extract. In parallel, the 800 g pellet was washed and re-suspended. Following further centrifugation at 2000 g for 10 minutes, the supernatant was decanted to give the nuclear extract. Aliquots were then incubated, in triplicate, for 16 hours with [17 α -methyl-³H]mibolerone and a 500 times higher concentration of triacrinolone acetonide. A second set of triplicates was incubated with excess of non-radioactive competitor at a 1000 times higher concentration in order to determine non-specific binding. Both cytosolic and nuclear extracts were stripped of free steroids using dextran-coated charcoal. Total binding sites were then measured.

Student's t-test was used for statistical analysis.

Endpoints/data interpretation

Total binding sites: measured and expressed as fmoles of [17 α -methyl-³H]mibolerone specifically bound per prostate.

The natural androgen dihydrotestosterone, and the synthetic progestins medroxyprogesterone acetate and norethisterone, were able to produce maximal levels of androgen receptors in the nuclear extracts by one hour after treatment. Other steroids did not show this effect. When progestin was administered one hour before dihydrotestosterone, the effects of dihydrotestosterone were markedly reduced by nomegestrol acetate, cyproterone acetate and megestrol acetate.

Prostatic weight or DNA/RNA content

Chen *et al.* (1988) studied the effects of a number of anti-androgenic chemicals on the weight and DNA content of the prostate of intact rats. Chemicals investigated included testosterone, cimetidine, progesterone, cannitracin and tolazoline. The intention was to establish a method that was both precise and reproducible.

Method

Immature male albino Sprague-Dawley rats were randomly assigned to treatment groups of six to 20 animals, as follows:

Group	Treatment	Dosage (mg/kg/day)
1	Vehicle (sesame oil)	0.2
2	TP	0.3
3	TP + cimetidine	0.3+20
4	TP + progesterone	0.3+10
5	TP + cannitracin	0.3+10
6	TP + tolazoline	0.3+7.5
7	TP + cimetidine + cannitracin	0.3+20+10

TP = testosterone propionate

Cannitracin was given by oral gavage. The other chemicals were administered by subcutaneous injection daily for seven days. Animals were killed 24 hours after the last administration, weighed, and the sex hormones removed and chilled. The ventral prostate and seminal vesicles were weighed and the ventral prostate either fixed in formalin for histological examination or retained for biochemical analysis, as appropriate.

Biochemical processing involved the mincing of tissue and preparation of a suspension. This was centrifuged at 1000 g for 10 minutes. RNA was removed from the pellet thus formed by alkaline hydrolysis. After re-precipitation, DNA in the pellet was acid hydrolysed. The DNA suspension was then centrifuged and the supernatant analysed for DNA using the reformed diphenylamine method with nucleoprotamine DNA as a reference material. RNA content was also assessed using the orcinol reaction with yeast RNA as standard.

Subsequent development

Phillips *et al.* (1990) investigated the androgenic activity of an oral contraceptive, norethindrone, using a castrated rat model. In this study, immature rats were castrated and given the test material by daily oral gavage for seven days; other animals similarly received the test material and a daily subcutaneous injection of testosterone propionate. On the eighth day of study, animals were humanely killed and the ventral prostate weight recorded.

Kuhnz and Beier (1994) investigated the potential androgenic activity of norgestimate and levonorgestrel using a similar castrated rat model. Castrated animals were assigned to 13 groups, six rats per group, and were treated with vehicle alone, either test material or testosterone propionate (positive control), for a 13-day treatment period. Animals were weighed on days 1 and 13 of treatment and then humanely killed on the day after the last dose. Seminal vesicles and prostate weights were recorded. Data were analysed by Dunnett's t-test.

Endpoints/data interpretation

- **Prostate and seminal vesicle weight:** expressed as bodyweight-relative organ weight.
- **DNA and RNA content:** measured by biochemical methods (see above) and expressed as total $\mu\text{g}/100$ g bodyweight (Chen *et al.*, only).

Chen *et al.* found that testosterone treatment markedly increased the weight of the ventral prostate and seminal vesicles. Animals receiving cimetidine, progesterone, cannitracin or tolazoline showed a marked reduction in organ weight when compared with those receiving testosterone alone, although values were still above those of the untreated controls. Prostatic RNA and DNA showed a significant decrease for rats receiving cimetidine compared to the testosterone-treated animals, whilst a less marked effect was seen for those given cannitracin. RNA content was reduced for the progesterone-treated group. The authors commented that anti-androgens generally elicited their effect by direct competition with androgens at the target organs and can directly inhibit the effect of exogenous testosterone in inducing growth of accessory sex organs. The authors believed this study also demonstrated that RNA and DNA contents could be used as precise and reliable markers.

In the study by Phillips *et al.* norethindrone was considered not to have any anti-androgenic activity on the basis of the lack of effect on prostatic weight. In contrast, Kuhnz and Beier established the androgenic activities of norgestimate and levonorgestrel, with clear evidence of a dosage-related increase in relative-organ weights being recorded for each chemical; norgestimate was, however, noted as possessing the greater activity.

Marker enzymes

Fjösne *et al.* (1992) developed a short-duration *in vivo* model to study the inducibility of ornithine decarboxylase (ODC) and S-adenosyl-methionine decarboxylase (AMDC) activities in the prostate and seminal vesicles. The model compared activities of androgen-stimulated castrates with those of untreated castrated animals and normal controls.

Method

Mature Wistar rats were castrated or sham-operated trans-scrotally. After a 10-day recovery period, animals were assigned to groups which received a single subcutaneous injection of either testosterone, at one of a series of dosages, or vehicle alone. After 24 hours, animals were exsanguinated under anaesthesia and the lobes of the prostate and the seminal vesicle removed. The tissues were homogenised in ice-cold buffer and ultracentrifuged. The supernatant was then incubated with either S-adenosyl-L-(carboxyl- ^{14}C) methionine or (1- ^{14}C) ornithine hydrochloride. The $^{14}\text{CO}_2$ evolved was trapped with potassium hydroxide and measured by scintillation counter. Assays were run in triplicate.

Data were analysed by Student's t-test.

Endpoints/data interpretation

- **Enzyme activity:** measured on the basis of radiolabelled carbon dioxide production, expressed as nanomoles $^{14}\text{CO}_2$ per hour per milligram of protein.

ODC activity in the intact rat was highest in the ventral prostate and lower in the dorsal prostate, coagulating gland and seminal vesicles; levels were hardly detectable in the lateral prostate. Following castration, enzyme levels were markedly lower in these tissues. Treatment of castrated animals with testosterone caused activity to increase, with 300 µg testosterone/100 g bodyweight giving half-maximal levels and 600 µg testosterone/100 g bodyweight giving a maximal response. For AMDC, activities were greatest in the ventral and dorsal prostate of intact males. Although castration resulted in a lowering of levels, they remained detectable. Administration of testosterone to castrated animals increased AMDC activity in all tissues, although the response to increasing doses was complex and varied between tissues.

The dose-response curve for ODC was steep and almost linear between 50 and 600 µg testosterone/100 g bodyweight, which suggested that it might be a suitable marker for bioassay of chemicals for anti-androgenic activity.

**IN VITRO SYSTEMS RELEVANT TO ASSESSMENT OF POTENTIAL TOXICITY TO
HUMANS – OESTROGEN RELATED**

MCF-7 CELL LINE SYSTEMS

MCF-7 cells can be used to assess oestrogenic activity through the ability to stimulate oestrogen receptor-dependent transcription and the promotion of growth of oestrogen-dependent cells in culture. Brooks *et al.* (1973) were the first to demonstrate a 17β -oestradiol binding protein, the oestrogen receptor, in a stable human cell line (MCF-7), derived by pleural effusion from a breast cancer patient. Soto and Sonnenschein (1985) used the C7MCF7-173 clone to examine the effects of 17β -oestradiol and human serum on cell proliferation in this system, whilst Welshons *et al.* (1987) evaluated the oestrogenic and anti-oestrogenic activity of the two phenolic lignans enterolactone and enterodiol, and also equol, in four assays in tissue culture including MCF-7 cells.

Subsequently, Pons *et al.* (1990) described a model to detect oestrogenic and anti-oestrogenic activity by bioluminescence in which MCF-7 cells were stably transfected with a reporter gene which allows expression of the firefly luciferase enzyme under control of the oestrogen regulatory element of the *Xenopus vitellogenin A2* gene.

Watanabe *et al.* (1990) examined the sensitivity of MCF-7 cells and other cell lines to 17β -oestradiol, tamoxifen and torimefine in a so-called miniaturised, improved nucleic acid precursor incorporation (MINI) assay.

Welshons *et al.* (1990) reported on the use of the oestrogen-responsive proliferation of MCF-7 cells as a bioassay for detection of dietary oestrogens. Oestrogenic activity of methanolic extracts of livestock feedstuffs was assessed in the assay. The assay was calibrated against a number of oestrogens and phytoestrogens, including oestradiol, diethylstilboestrol, zearalenone, formononetin, genistein, daidzein, biochanin-A and coumestrol. Mayr *et al.* (1992) used the oestrogen-dependent induction of an exoprotein in MCF-7 cells to assess the oestrogenic activity of zearalenone, phytoestrogens (including coumestrol, genistein, daidzein, biochanin-A and formononetin) and extracts of cereals.

In a series of papers, Soto *et al.* have promoted the use of this cell line as a screening tool. Soto *et al.* (1991) identified nonylphenol as an oestrogenic chemical released from plastic centrifuge tubes which induced cell proliferation and progesterone receptor in MCF-7 cells. This was followed in 1992 by a paper by Soto *et al.* which described an MCF-7 cell culture bioassay to assess the oestrogenicity of xenobiotics (the so-called E-Screen). Chemicals tested in this system included Arochlor 1221, chlordane, chlordecone, heptachlor, mirex, *o,p'*-DDT, a number of alkylphenols (including nonylphenol), and coumestrol and zearalenone. Soto *et al.* (1995) reported further on the development of the E-screen. Compounds reported to be positive in this system included some alkylphenols, phthalates, PCB congeners, hydroxylated PCBs, and the insecticides dieldrin, endosulfan and toxaphene.

Miksicek (1993) tested the oestrogenic activity of a number of plant flavonoids (including flavone, apigenin, naringenin, zearalenone, genistein, chalcone and 4, 4'-dihydroxychalcone) using MCF-7 cell proliferation as an endpoint. Jaing *et al.* (1993) used MCF-7 cells to assess the effects on proliferation of the anti-oestrogens keoxifene and RU 39411 with reference to that of oestradiol. Berthois *et al.* (1994) compared the immunoreactivity of MCF-7 cell oestrogen receptors, when bound to a number of anti-oestrogens or oestrogens to investigate possible mechanisms, to explain the dual agonist/antagonist behaviour of anti-oestrogens. Harper *et al.* (1994) investigated the effects of oestradiol and aryl hydrocarbon receptor agonists, including dioxin and other related compounds, on progesterone receptor (PR) binding, nuclear PR formation, and PR mRNA levels in MCF-7 cells.

In an abstract, von Angerer *et al.* (1994) described the use of MCF-7 cells transiently transfected with a reporter plasmid containing the luciferase gene under the control of an oestrogen response element for evaluating the oestrogenic activity of various compounds. A further paper by the same authors used these transiently transfected MCF-7 cells to test the effects of an oestrogenic drug (Meyer *et al.*, 1994). In a 1995 paper, Desaulniers *et al.* compared various proliferation indices of MCF-7 cells (including the MTT assay, [³H]thymidine incorporation, DNA assay and counting cell nuclei) and tested the effects of different culture conditions on cell proliferation.

vom Saal *et al.* (1995) investigated the ability of *o,p'*-DDT and methoxychlor to compete with oestradiol for binding to oestrogen receptors in MCF-7 cells in both serum-free medium and 100% serum. Zacharewski *et al.* (1995) described the use of two different assays involving MCF-7 cells to detect oestrogenic activity in pulp and paper mill black liquor and effluent. In one assay, MCF-7 cells were transiently transfected with a recombinant oestrogen receptor and a luciferase reporter gene; in the other assay, MCF-7 cells, were transiently transfected with the pS2Luc reporter gene to investigate the ability of the effluents to suppress oestrogen-induced luciferase activity through induction of pS2, an oestradiol-inducible protein secreted by MCF-7 cells.

Connor *et al.* (1996) reported on the assessment of the herbicides atrazine and simazine for oestrogenic activity in MCF-7 cells, using induction of cell proliferation and formation of nuclear progesterone receptor-DNA complexes as endpoints. Additional experiments were carried out using MCF-7 cells transiently transfected with a Gal4-human oestrogen receptor chimera and a chimeric receptor-regulated luciferase reporter gene to assess the ability of these chemicals to induce luciferase activity. Mellanen *et al.* (1996) tested several wood-derived compounds, including β -sitosterol and abietic acid, for their effect on MCF-7 cell proliferation.

Method

Brooks *et al.* (1973): A primary culture of human breast carcinoma cells was obtained by pleural effusion from a female patient with metastatic disease. A stable epithelioid cell line, MCF-7, was derived from free-floating passages of these cells. The MCF-7 cells were maintained in minimal essential medium (MEM) supplemented with non-essential amino acids, insulin, antibiotics and calf serum. Following growth of the cells to confluence, a microsome-free supernatant was prepared. A saturation curve for 17 β -oestradiol was generated by incubating aliquots of supernatant with different concentrations of [³H]17 β -oestradiol, passing down a Sephadex G-25 column, and assessing radioactivity by liquid scintillation. The density gradient pattern of cytoplasmic 17 β -oestradiol binding protein was assessed by sucrose gradient sedimentation. Inhibition of [³H]17 β -oestradiol binding to the receptor was investigated by assessing radioactivity of aliquots incubated with unlabelled oestradiol followed by addition of varying concentrations of [³H]17 β -oestradiol. Migration of cytoplasmic [³H]17 β -oestradiol receptor complex into the nucleus was assessed in an experiment in which two aliquots of cells were incubated at either 0°C or 37°C with [³H]17 β -oestradiol followed by a further incubation with unlabelled oestradiol and measuring of radioactivity in the cytoplasmic and nuclear fractions.

Soto and Sonnenschein (1985): Cell proliferation was assessed in cultures of C7MCF7-173 cells by harvesting cells in the exponential growth phase and counting them using a Coulter counter. Results were expressed as the mean generation time (time in which an exponentially growing culture doubles in size) in hours, or as proliferation rates when many variables were compared in one experiment. The effect of 17 β oestradiol on C7MCF7-173 cell proliferation was tested in serum-free medium. To test the effect of

serum on the growth of these cells, heat-inactivated serum (stripped using dextran-coated charcoal) from healthy cycling women at days 0-14 of their menstrual cycle, adult men, and post-menopausal women was used. The binding profile of oestrogen receptors ("estrophilins") was determined using a sedimentation method.

Welshons *et al.* (1987): MCF-7 cells were maintained in MEM with calf serum and transferred to phenol red-free medium five to seven days before the experiments. Cultures were treated for three to four days following a period of one to three days for cell attachment with daily medium changes. Effects were measured on cell growth (measured as DNA content using a fluorometric method with Hoechst dye 33258) and on levels of progesterone receptors. The latter involved incubation of cells with [³H]R5020 or unlabelled R5020 (to measure non-specific binding) followed by measurement of radioactivity and/or DNA.

Pons *et al.* (1990): The authors described the transfection of MCF-7 cells with a pVit-tk-Luc plasmid containing the *Xenopus vitellogenin A2* gene (which contains an oestrogen-responsive element) inserted in front of the herpes simplex virus promoter for thymidine kinase, which controls the firefly luciferase structural gene. Transfection was carried out using the calcium phosphate precipitation technique. The plasmid pVit-tk-Luc and another plasmid, pAG-60 (containing the bacterial aminoglycoside phosphotransferase gene under control of the thymidine kinase promoter, expression of which confers resistance to the antibiotic geneticin), were co-precipitated with calcium chloride and added to MCF-7 cells in culture for 18-24 hours. Cells were then replated into multi-well dishes and cultured in the presence of geneticin. Resistant clones were selected such that they retained their expression of a specific luciferase activity in the presence of oestradiol, which was abolished with 100-fold excess 4-hydroxytamoxifen. Using a single-photon detecting camera, luciferase activity was detected in individual whole cells, allowing the selection of the more luminescent sub-clones.

For the experiments, cells were maintained in medium containing 1% fetal calf serum (stripped using dextran-coated charcoal) and allowed to attach for 24 hours. Following this, the medium was replaced with fresh medium containing various concentrations of oestradiol in the presence or absence of different concentrations of 4-hydroxytamoxifen and cells were incubated for 24 and 48 hours before assessment of luciferase activity either in whole cells or in cell-free conditions.

Whole cell luciferase activity was assessed by adding luciferin to the culture medium following treatment, and immediately introducing the dishes into a dark room for measurement of luminescence by the single-photon camera. Cell-free luciferase activity was determined by harvesting cells into tubes containing luminescence buffer, sonicating the tubes, placing them in a luminometer, and automatically injecting each tube with luciferin.

Watanabe *et al.* (1990): The concentrations of oestrogen and progesterone receptors in the cytosol of MCF-7 cells were determined using enzyme immunoassay kits. The miniaturised, improved nucleic acid precursor incorporation assay (MINI assay) was performed as follows: a 0.6% agarose layer was prepared in each well of 24-well plates and allowed to harden, after which a 0.4% agarose solution containing the MCF-7 cells was overlaid and itself allowed to harden. Culture medium (phenol red-free MEM with 10% calf serum stripped using dextran-coated charcoal) containing the test substance was added to the top of these layers and plates were incubated for four days, followed by addition of tritiated thymidine and a further 24-hour incubation. Cell pellets were prepared by centrifugation and, following sedimentation in serum albumin, the radioactivity in the resulting solution was counted using liquid scintillation.

Welshons *et al.* (1990): MCF-7 cell proliferation, measured as DNA content (see Welshons *et al.*, 1987) was used to assess the effect of known oestrogens (oestradiol, diethylstilboestrol and zearalenone), phytoestrogens (formononetin, genistein, daidzein, biochanin A and coumestrol) and methanol extracts of animal feeds. The oestrogenic activity of feed extracts was confirmed by competitive inhibition with the anti-oestrogens tamoxifen or LY156758 (keoxifene) and analysis for oestrogens, using tandem mass spectrometry with known oestrogen standards.

Soto *et al.* (1991): Human serum (stripped using dextran-coated charcoal) stored in new polystyrene tubes was found to no longer inhibit MCF-7 cell proliferation, as determined by counting cells using a Coulter counter (see Soto and Sonnenschein, 1985). Extracts of these and a different brand of tubes were tested in MCF-7 cells for their effects on proliferation and progesterone receptor induction. Cells were exposed to test substances for six days and progesterone receptors were determined by freezing cells, incubating with monothioglycerol, and using an Abbott progesterone receptor enzyme immunoassay.

Mayr *et al.* (1992): Zearalenone and a number of phytoestrogens dissolved in ethanol were tested for oestrogenic activity in MCF-7 cells, together with acetonitrile/water extracts of cereals (wheat, barley and oats). Cells were exposed for three days, cell proteins were labelled with [³⁵S]methionine, separated by SDS-polyacrylamide gel electrophoresis, and visualised by autoradiography. ED₅₀ values (the concentration required to produce a half-maximal response) for the test compounds were estimated by using serial dilutions and comparing the intensity of the 52-kDa band, corresponding to the oestrogen-specific exoprotein, with that induced by 17 β -oestradiol. The specificity of exoprotein induction was investigated by treating cells with progesterone, testosterone, tamoxifen (partial anti-oestrogen) and 1-(aminoalkyl)-5-hydroxy-2-(4-hydroxyphenyl)indol (pure anti-oestrogen).

Soto *et al.* (1992): Heat-inactivated human serum (stripped using dextran-coated charcoal) was added to MCF-7 cells in culture, together with a range of concentrations of test compounds. Cells were lysed on day 6, and proliferation was assessed by counting nuclei using a Coulter counter.

Miksicek (1993): MCF-7 cells were grown in medium containing charcoal-stripped calf serum and various supplements for up to 12 days, with medium changes every two days. Following this, the cells were dispersed and the number of viable cells was counted using a haemocytometer.

Jaing *et al.* (1993): Following two days of incubation in oestrogen-free medium, MCF-7 cells were cultured with medium containing test compounds for a period of five days. Cells were then removed and lysed. A fluorescence method using Hoechst dye 33258 was used to determine the level of total DNA as a measure of cell growth.

Berthois *et al.* (1994): MCF-7 cells maintained in medium containing calf serum (stripped using dextran-coated charcoal) were seeded into flasks in phenol red-free medium, followed after two days by addition of test chemicals ([³H]oestradiol, tamoxifen, trans-hydroxytamoxifen and Z-4-hydroxy[N-methyl-³H]tamoxifen). Media were replaced every two days, and cells were harvested and counted when still in a logarithmic growth phase (number of days not specified). In a separate series of experiments, cytosolic and nuclear oestrogen receptors were prepared using a number of homogenisation and centrifugation steps and levels of receptors were measured using a standard Abbott oestrogen receptor EIA. A modified procedure also allowed the measurement of both occupied and unoccupied receptors.

Harper *et al.* (1994): MCF-7 cells were exposed to test chemicals in culture for 72 hours, with medium changes every 48 hours. Following several centrifugation and washing steps, cells were homogenised and cytosolic extracts were prepared. Extracts were assayed for progesterone receptors (PR) using a

hydroxylapatite method involving incubation with [³H]promegestone followed by liquid scintillation counting. Cytosolic PR levels were quantitated by measuring the amount of radioactivity per mg of protein. Levels of immunoreactive PR in cytosol were measured using an Abbott PR EIA. Levels of nuclear PR binding of oestradiol in the presence and absence of dioxin were determined following three-day treatments by velocity sedimentation analysis using a 5-25% sucrose density gradient. Levels of PR mRNA were determined following a three-day treatment with oestradiol, dioxin or the combination by Northern blot analysis. Total RNA was extracted by the guanidinium thiocyanate/acid phenol method, followed by affinity purification of Poly(A⁺)RNA. This was separated on an agarose gel, transferred to a nylon membrane, and bound by UV crosslinking. Following a pre-hybridisation step, the membrane was hybridised with [³²P]CTP-labelled cDNA probes for the human PR for 24 hours, stripped, reprobbed, visualised by autoradiography, and quantitated by scanning on a blot analyser.

von Angerer *et al.* (1994) and Meyer *et al.* (1994): The authors described the transient transfection of MCF-7 cells with a reporter plasmid (pERE luc) containing the luciferase gene under the control of an oestrogen response element and derived from pGEM/luc and EREwtc. MCF-7 cells were transfected using the calcium phosphate/DNA co-precipitation method and transfection efficiency was determined by co-transfection of the reference vector pCH110 and analysis of β -galactosidase activity. Cells were treated with test compounds approximately five hours after transfection for a period of one hour, followed by a further three and a half hour incubation in fresh medium and subsequent assay of luciferase activity. In this system, luciferase activity was stimulated by oestrogens whereas anti-oestrogens abolished the oestrogen-induced activity.

Desaulniers *et al.* (1995): A number of endpoints to assess MCF-7 cell proliferation were compared, including the MTT assay, [³H]thymidine incorporation, DNA assay and counting of cell nuclei (no further details given). The effect of various culture conditions on cell proliferation were also examined (including concentrations of human sera and preconditioning treatment).

Soto *et al.* (1995): For cell proliferation experiments, following a 24 hour period for cell attachment, MCF-7 cells were grown in 5% human serum (stripped using dextran-coated charcoal) containing test compounds for a period of five days and the nuclei were then counted in a Coulter counter. Oestrogen and progesterone receptors were assayed in cells in another series of experiments, following a three-day treatment period by incubation in an extraction buffer, centrifugation, and measurement using Abbott oestrogen and progesterone EIA kits. Another marker of oestrogen action, induction of the protein pS2, was assessed in cells treated for three days in culture using a commercially available immunoradiometric assay.

vom Saal *et al.* (1995): Relative binding affinities (concentration of chemical required to displace 50% [³H]oestradiol from the oestrogen receptor relative to unlabelled oestradiol) of *o,p'*-DDT and methoxychlor were determined by incubating MCF-7 cells with a range of concentrations of the chemicals in the presence of [³H]oestradiol, either in serum-free medium or in 100% human serum from adult males (no further details given).

Zacharewski *et al.* (1995): For the assay of oestrogenic activity, MCF-7 cells cultured in media containing 5% fetal bovine serum (stripped using dextran-coated charcoal) were transfected with pCH110 (β -galactosidase expression vector used to correct for variations in transfection efficiency), 17m5-G-Luc, Gal-HEGO and pBS (carrier DNA). To assay for anti-oestrogenic activity, MCF-7 cells were transfected with pCH110, pS2Luc, HEGO and pBS. All transient transfections were performed using the calcium phosphate co-precipitation technique. Twenty-four hours after transfection, cells were treated with oestradiol, pulp and paper mill black liquor or effluent, or combinations of oestradiol and liquor/effluent

for a period of 24 hours. Luciferase activity (method of detection not detailed) was expressed as a percentage relative to the maximum induction observed by 1 nM oestradiol.

Connor *et al.* (1996): MCF-7 cells were treated in medium containing 5% fetal bovine serum (stripped using dextran-coated charcoal), together with oestradiol, atrazine and simazine and combinations of oestradiol and atrazine or simazine, for a period of 11 days with medium changes containing fresh test compound(s) every two days, following which cell numbers were assessed. When assaying for PR-response element complexes, cells were treated for three days, followed by the use of a gel electrophoretic mobility shift assay which involved the preparation of nuclei and extraction of PR complexes. Nuclear extracts were then incubated with a [³²P]-labelled progesterone response element/glucocorticoid response element oligonucleotide duplex, after which they were loaded on to a 5% non-denaturing polyacrylamide gel. Following drying, protein-DNA complexes were visualised by autoradiography and quantified.

For the transfection assays, MCF-7 cells were transiently transfected with a Gal4-human oestrogen receptor chimera and a chimeric receptor-regulated luciferase reporter gene using a calcium phosphate co-precipitation technique. Twenty-four hours after transfection, cells were incubated with test compound (oestradiol, atrazine, simazine or atrazine and simazine in combination) for 24 hours, following which cells were harvested and an assay for luciferase was performed. A reference β -galactosidase expression vector was co-transfected as an internal standard to correct for variations in transfection efficiency.

Mellanen *et al.* (1996): MCF-7 cells were seeded in medium containing 5% fetal calf serum (stripped using dextran-coated charcoal) for 24 hours to allow for cell attachment. Following this, medium was replaced with fresh medium containing test compounds (including β -sitosterol and abietic acid), cells were cultured for seven days with medium changes on alternate days, and cell proliferation was assessed.

Endpoints/data interpretation

Brooks *et al.* (1973):

- **Binding of [³H]17 β -oestradiol to oestrogen receptor, inhibition of labelled oestradiol binding to the cytoplasmic receptor and migration of bound [³H]17 β -oestradiol receptor complex:** all assessed by liquid scintillation counting.
- **Protein:** assayed by the Lowry method.

For the assessment of sedimentation constants, supernatant proteins were separated on a sucrose gradient following incubation with [³H]17 β -oestradiol and the radioactivity in the fractions was determined. A saturation binding curve was produced for the 17 β -oestradiol receptor in the microsome-free supernatant of MCF-7 cells. Results showed that the oestradiol-receptor complex migrated from the cytoplasm to the nuclei of these cells.

Soto and Sonnenschein (1985):

- **Cell numbers:** determined using a Coulter counter, with proliferation expressed as the time (in hours) for a culture in exponential growth to double in size.
- **Oestradiol levels:** measured using a radioimmunoassay technique. Sex steroids were removed from human sera by dextran-charcoal adsorption.

Oestradiol did not increase the cell proliferation rate over controls when added to serum-free media. Media supplemented with sex steroid-stripped human female serum resulted in inhibition of cell proliferation in a concentration-dependent fashion. Addition of 17β -oestradiol to these cells significantly increased proliferation rates, although the concentration required to produce a maximal rate increased with increase in serum concentration. Addition of growth factors (*e.g.* insulin, EGF) and non-oestrogenic steroids failed to overcome the inhibitory effect of serum.

Welshons *et al.* (1987):

- **DNA content:** assessed using Hoechst 33258 and fluorometric determination, to assess cell growth.
- **Progesterone receptor levels:** measured using tritiated R5020 for specific binding or unlabelled R5020 to measure non-specific binding. The method of assessing of radioactivity was not given.

Enterolactone, enterodiol and equol showed weak oestrogenic properties in the assay, measured as increases in levels of progesterone receptors relative to oestradiol and also as stimulation of cell growth. The stimulation of both endpoints by these compounds was inhibited by the anti-oestrogen tamoxifen.

Pons *et al.* (1990):

- **Luciferase activity:** determined in cell-free conditions using a luminometer and integrating the value of the luminescence peak following luciferin injection.
- **Protein levels:** determined by the Lowry method and activity expressed as pg luciferase using a standard curve with pure enzyme.
- **Luciferase activity:** determined in whole cells using a single-photon-detecting camera for luminescence measurement. Light intensity was monitored for 1-10 minutes, quantified per unit area and compared to a standard probe (a closed tube containing a ^{14}C compound in scintillation liquid) placed nearby.

A sub-clone of stable transfectants of MCF-7 cells (termed MVLN-15-C7 cells) was produced containing the plasmid pVit-tk-Luc. Using a sub-cloning step, cells were found to maintain their level of expression of luciferase activity in response to oestradiol after 20 passages (an eight-month culture). The sensitivity to oestradiol, measured as the EC_{50} , was 20-30 pM. Luciferase activity induced by oestradiol (1 nM) was significantly inhibited by 100 nM 4-hydroxytamoxifen and completely inhibited by 1 μM . The compounds dexamethasone, progesterone, testosterone, aldosterone and retinoic acid had no significant effect on induction of luciferase. Luciferase measurement in whole cells demonstrated the oestrogen dependence of the luminescence and was reversible on addition of 4-hydroxytamoxifen.

Watanabe *et al.* (1990):

- **Oestrogen and progesterone receptors:** measured using enzyme radioimmunoassay kits.
- **Protein levels:** determined using the Coomassie brilliant blue method (Bio-Rad kit).
- **Growth:** assessed using the MINI assay, which measures the radioactive incorporation of tritiated thymidine after a four-day incubation. Radioactivity was determined by liquid scintillation counting.

Oestradiol was shown to enhance thymidine incorporation by MCF-7 cells at concentrations as low as 10^{-11} M. Tamoxifen (10^{-5} M) suppressed thymidine incorporation to 10% of the control level, whereas for toremifene 50% suppression was not observed even at 10^{-5} M.

Welshons *et al.* (1990):

- **DNA content:** see Welshons *et al.*, 1987.

Presence of known oestrogens in extracts of feedstuffs was confirmed by tandem mass spectrometry. The mass spectrometer was operated on the electron impact ionisation and daughter ion modes, using argon as the collision gas. Voltages were set specifically for each oestrogen tested. Oestrogens and oestrogenic feedstuffs stimulated MCF-7 cell proliferation, measured as DNA content, compared with controls. Oestradiol was the most potent, followed by zearalenone and then the phytoestrogens, of which coumestrol was the most potent. A total of 166 feed samples were tested, and 88 showed detectable oestrogenic activity (76 of these had activity <1 ppm equivalent of zearalenone). One sample showed activity >10 ppm zearalenone equivalents and was shown to contain formononetin, coumestrol and biochanin A.

Soto *et al.* (1991):

- **Cell proliferation:** assessed following six days in culture by lysing the cells and counting the nuclei in a Coulter counter, expressed as cells/well.
- **Progesterone receptor induction:** assessed by freezing cells following treatment and determining the levels of progesterone receptors in extracts using an Abbott enzyme immunoassay.

p-nonylphenol was identified as an oestrogenic substance, present in plastic centrifuge tubes, which induced both MCF-7 cell proliferation and progesterone receptor induction.

Mayr *et al.* (1992):

- **Oestrogenic activity:** assessed by measuring the induction of an oestrogen-specific 52-kDa exoprotein by radiolabelling the cell proteins using [³⁵S]methionine, separating these by SDS-polyacrylamide gel electrophoresis, and visualisation by autoradiography. By comparing the intensity of the band with that induced by oestradiol, ED₅₀ values were estimated.

Relative to oestradiol, the mycoestrogen zearalenone was the most potent (3-5% relative potency), with coumestrol (0.03%) and genistein (0.01%) approximately 10 times less potent, and genistin (0.005%), daidzein (0.002%), daidzin, biochanin-A and formononetin (0.001%) almost another order of magnitude less potent. No effect on exoprotein induction was shown with progesterone, testosterone or the pure anti-oestrogen, whilst tamoxifen produced a weak induction at the maximum dose tested. In 12 of the 15 cereal extracts tested, the oestrogenic activity correlated with the levels of zearalenone, as analysed by HPLC.

Soto *et al.* (1992):

- **Cell proliferation:** assessed by counting cell nuclei using a Coulter counter and expressing the results as
(a) the relative proliferative potency (RPP, the ratio between the minimal concentration of oestradiol needed for maximal cell yield and the minimal concentration of test compound required to achieve the same result), and
(b) the relative proliferative effect (RPE, 100× the ratio between the highest cell yield obtained with the chemical and that obtained with oestradiol).

An RPE of 100 indicates full agonist activity, whilst an RPE significantly below that obtained with oestradiol indicates partial agonist activity.

Relative to oestradiol (assigned 100% activity), RPE/RPP values for the test compounds were diethylstilboestrol (112%/1000%), zearalenone (88%/1%), coumestrol (93%/0.001%), chlordecone (81%/0.0001%), *p,p'*-DDT (70%/0.0001%), *o,p'*-DDD (84%/0.0001%) and 4-nonylphenol (100%/0.001%).

Miksicek (1993):

- **Total cell numbers per culture dish:** averaged from duplicate determinations, counted using a haemocytometer.

At 1 μ M concentrations, apigenin and 4,4'-dihydroxychalcone had equivalent stimulatory effects on MCF-7 cell proliferation as 10 nM oestradiol. Neither flavone (1 μ M) nor the oestrogen antagonist ICI-164,384 (1 μ M) had any stimulatory effect on cell proliferation in this assay.

Jaing *et al.* (1993):

- **Total DNA:** measured following growth with medium containing test compounds for a period of five days, determined by a fluorescence method using the dye Hoechst 33258.

The lowest concentration of oestradiol required for maximal stimulation of the growth of MCF-7 cells was 0.1 nM. Both antagonists, keoxifene and RU 39411, had weak agonist effects on cell growth at 0.1 nM, but at 10 nM exhibited anti-oestrogenic activities by completely reversing the growth regulatory effect of 0.1 nM oestradiol.

Berthois *et al.* (1994):

- **Cell proliferation:** assessed in subconfluent cultures by harvesting cells and counting using a cell counter.
- **Cytosolic and nuclear oestrogen receptors:** measured using an Abbott oestrogen receptor EIA. Using a modification of the EIA, involving an exchange procedure to measure radioligand binding, allowed the measurement of both occupied and unoccupied receptors.

The anti-oestrogens tamoxifen and hydroxytamoxifen displayed mixed partial agonist/antagonist activity on MCF-7 cell proliferation. At low concentrations they stimulated cell proliferation, whilst at higher levels no stimulation was shown. It was proposed that agonist activity of anti-oestrogens could be due to a receptor complex containing anti-oestrogens primarily in the oestrogen-binding sites, whilst antagonist activity could depend on the occupation of additional sites on the receptor that does not bind oestradiol.

Harper *et al.* (1994):

- **Cytosol PR levels:** determined by a hydroxylapatite assay using radiolabelled promegestone and liquid scintillation counting.
- **Immunoreactive PR levels:** determined using an Abbott EIA monoclonal antibody kit.

- **PR binding studies:** carried out using velocity sedimentation analysis on a 5-25% sucrose density gradient, separating the gradients into 30 fractions and determining the radioactivity of each fraction by liquid scintillation counting.
- **PR mRNA levels:** determined by Northern blot analysis using radiolabelled cDNA probes for human PR.

Oestradiol induced PR binding, immunoreactive PR levels, nuclear PR formation and PR mRNA levels in MCF-7 cells. Dioxin (1 nM) had no effect on these responses although, when co-treated with oestradiol, dioxin significantly inhibited all the oestradiol-induced responses and decreased the number of oestradiol-induced PR cellular binding sites. 3-Methylcholanthrene, a prototypical polycyclic aromatic hydrocarbon (PAH), also inhibited oestradiol-induced PR binding and immunoreactive PR levels. Using other PAHs, potency for this inhibition paralleled binding of PAHs to the aryl hydrocarbon receptor.

von Angerer *et al.* (1994) and Meyer *et al.* (1994):

- **Oestrogenic responses:** detected by the measurement of luciferase activity in transiently transfected MCF-7, cells using a commercial assay kit and measuring luminescence for 10-second periods in a luminometer. Activity was standardised to protein content and expressed as fg luciferase/ μ g protein by referring to a luciferase/luminescence standard curve.
- **Protein determination:** with the Bradford method for detecting μ g quantities, utilising the principle of protein-dye binding.

Luciferase activity was stimulated by oestrogens in this test system, whilst anti-oestrogens inhibited the oestrogen-induced stimulation.

Desaulniers *et al.* (1995):

- **Indices of cell proliferation:** DNA assay, MTT assay, cell counting using a Coulter counter, and [3 H]thymidine incorporation.

The DNA assay was found to be the least sensitive, followed by the MTT assay. Counting cell nuclei using a Coulter counter generated more variable results than [3 H]thymidine incorporation, which was the most sensitive proliferation index. Cells grown in medium supplemented with 15% human serum produced an optimal response to oestradiol and preconditioning cells for five days in medium without oestradiol before seeding improved subsequent oestradiol-induced proliferation.

Soto *et al.* (1995):

- **Cell proliferation:** assessed by counting cell nuclei in a Coulter counter.
- **Oestrogen and progesterone receptor levels:** assayed using Abbott oestrogen and progesterone EIA kits.
- **pS2 levels:** assayed using a commercially available immunoradiometric assay.

Using the E-screen, several chemicals were identified as having oestrogenic activity (defined as promoting cell proliferation), including some alkylphenols, phthalates, PCB congeners, hydroxylated PCBs, and the insecticides dieldrin, endosulfan and toxaphene. In addition, these compounds competed with oestradiol for binding to the oestrogen receptor and increased levels of progesterone receptors and pS2 protein. Recombinant human growth factors and insulin did not induce cell proliferation.

vom Saal *et al.* (1995):

- **Relative binding affinities:** RBA of *o,p'*-DDT and methoxychlor for the oestrogen receptor were calculated in MCF-7 cells by incubating the cells with a saturating concentration of [³H]oestradiol in either serum-free medium or 100% human serum and then adding a range of concentrations of unlabelled oestradiol or the test chemicals. The RBA was defined as the ratio of the concentration of unlabelled oestradiol required to displace 50% of [³H]oestradiol divided by the concentration of chemical required to do this.

Saturation binding profiles of [³H]oestradiol in serum-free medium and 100% serum indicated that approximately 95% of the oestradiol was bound in human serum. The RBA of *o,p'*-DDT was greater in serum than in serum-free medium, whereas the RBA for methoxychlor was similar in both media.

Zacharewski *et al.* (1995):

- **Oestrogenic activity:** detected by the measurement of luciferase activity in MCF-7 cells transiently transfected with 17m5-G-Luc and Gal4-HEGO (no further details given).
- **Anti-oestrogenic activity:** detected by the measurement of luciferase activity in MCF-7 cells transiently transfected with pS2Luc and HEGO (no further details given).

MCF-7 cells, transfected with Gal4-HEGO and 17m5-G-Luc, demonstrated dose-dependent increases in luciferase activity following treatment with pulp black liquor. Based on the EC₅₀ of 20 pM, the detection limit of the assay for oestradiol was approximately 5 pg/mL. Cells transiently transfected with 17m-G-Luc alone did not show increased luciferase activity following exposure to oestradiol, the black liquor or the effluent. Co-treatment experiments with oestradiol and black liquor caused a significantly higher induction than that observed with oestradiol alone. Pulp and paper mill effluent had no significant effect on oestradiol-induced luciferase activity. Treatment of cells with ICI-164,384, a pure anti-oestrogen, abolished the induction of luciferase activity by oestradiol, black liquor and oestradiol and liquor combined. MCF-7 cells transiently transfected with pS2Luc showed a six-fold induction of luciferase activity following treatment with 1 nM oestradiol. Cells co-treated with oestradiol and paper and pulp effluent showed a reduction in the oestradiol-induced activity. Treatment with effluent alone had no effect on luciferase activity in these cells.

Connor *et al.* (1996):

- **Cell proliferation:** assessed after a treatment period of 11 days by counting cells using a Coulter counter.
- **Identification of nuclear PR-DNA complexes:** a gel electrophoretic mobility shift assay incorporating a [³²P]-labelled progesterone response element/glucocorticoid response element oligonucleotide duplex and loading on to a 5% non-denaturing polyacrylamide gel. Complexes were visualised by autoradiography and quantified with a blot analyser.

- **Oestrogenic responses:** detected by measurement of luciferase activity in transiently transfected MCF-7 cells (no further details given).

Neither atrazine nor simazine significantly altered MCF-7 cell proliferation at any concentration tested, and neither had an effect on oestradiol-induced proliferation. Also, neither significantly induced the formation of PR-DNA complexes in nuclear extracts of MCF-7 cells or had any effect on the intensity of oestradiol-induced complex formation. In addition, neither chemical significantly induced reporter gene activity in transiently transfected MCF-7 cells and neither inhibited or enhanced oestradiol-induced activity.

Mellanen *et al.* (1996):

- **Cell proliferation:** assessed following a seven-day treatment period using a colourimetric MTT assay.

Abietic acid was oestrogenic in the MCF-7 cell proliferation assay, whereas β -sitosterol was negative. β -sitosterol was found to be oestrogenic in another oestrogen-dependent cell line (T-47D), suggesting that it may have to be metabolised to a hormonally active metabolite.

ISHIKAWA CELL LINE SYSTEMS

A number of systems using Ishikawa human endometrial cancer cells are reviewed below. These were reported by Holinka *et al.* (1989), Littlefield *et al.* (1990) and Markiewicz *et al.* (1993).

Holinka *et al.* (1989): Previous work by the authors had used the Ishikawa cell line to investigate effects of oestradiol. The test system then in use involved the use of serum. This was regarded as a weakness, because the serum could contain endogenous chemicals which might influence or mediate the action of oestradiol. In this paper, work was presented on the development and validation of a test system that did not require culture with serum, and on various endpoints that could be used to assay chemical activity.

Littlefield *et al.* (1990): In this paper a novel assay was presented in which the quantitation of alkaline phosphatase activity by Ishikawa cells is an endpoint for oestrogenic activity by a xenobiotic.

Ignar-Trowbridge *et al.* (1993) investigated the effect of peptide growth factors on the activation of a consensus oestrogen-responsive element using transfected Ishikawa cells.

Markiewicz *et al.* (1993): The results of studies on a series of non-steroidal phytoestrogens, steroids and anti-oestrogens were presented, using the same endpoint as the Littlefield *et al.* (1990) study. Chemicals examined were oestradiol, formononetin, biochanin A, 4-hydroxytamoxifen, [N-n-butyl-N-methyl-11-(3,17 β dihydroxyestra-1,3,5-(10)-trien-7 α -yl) undecanamide], daidzein, equol and genistein.

METHOD

Holinka *et al.* (1989): The Ishikawa cells were maintained under oestrogen-free conditions for at least two weeks before experimentation. The effects of oestrogen and serum were investigated, either alone or in combination using a number of endpoints.

In order to evaluate effects on proliferation, the cells were seeded (50,000/dish) in basal medium in the presence of charcoal-treated serum. On the following day the medium was exchanged for one containing either the vehicle alone, oestradiol (the test material) in vehicle, serum, or oestradiol and serum. Media were changed every two days. At intervals throughout an 11-day period cells were counted. These conditions also permitted study of effects on the exponential growth rate of cells. The effect on colony formation efficiency of incubation in basal or serum-containing media, with or without exogenous oestradiol, was investigated by seeding cells at densities from 10,000 to 100,000/dish and counting colonies greater than 50 μ m in diameter. Labelling index was investigated by exposing cells to [3 H]thymidine for 70 minutes during the exponential growth phase, harvesting by exposure to trypsin-EDTA, and spreading on a slide. Slides were then autoradiographed. The fraction of labelled cells was determined after staining with safranin O. The metaphase index was estimated from the exponentially growing cells by exposure to colchicine, harvesting, fixing, and visualisation with Giemsa under light microscopy. Statistical significances were assessed by Students' t-test.

Littlefield *et al.* (1990): Ishikawa cells were maintained under standard culture conditions, with twice-weekly passage. Twenty-four hours before commencement of study, near-confluent cells were changed to an oestrogen-free basal medium from which oestrogens had been removed through passage over dextran-coated charcoal. At the start of a study, cells were plated in a 96-well, flat-bottomed microtitre plate in oestrogen-free medium at a density of 2.5×10^4 cells/well. The cells were incubated with ethanol (vehicle

control) or the test chemical was dissolved in ethanol for 72 hours. All treatments were performed in quadruplicate. At the conclusion of the exposure period the growth medium was removed from the microtitre plate, which was thoroughly washed in a solution of sodium phosphate and sodium chloride. The plate was deep-frozen (-80°C) for 15 minutes, then thawed to room temperature for five to ten minutes, placed on ice, and an ice-cold solution of p-nitrophenyl phosphate and diethanolamine added. After warming to room temperature, the yellow colouration from p-nitrophenol was allowed to develop. Plates were monitored periodically until maximally stimulated cells showed an absorbency of about 1.2 at 405 nm. Effects of various natural and synthetic oestrogens, anti-oestrogens, androgens, glucocorticoids, mineralocorticoids and $\Delta 5\text{-}3\beta\text{-hydroxy}$ steroids were investigated.

Ignar-Trowbridge *et al.* (1993): The ability of peptide growth factor to activate transcription from a consensus oestrogen-response element (ERE) in an oestrogen receptor (ER)-dependent manner was assessed using transfection techniques on Ishikawa cells having negligible levels of ER. Ishikawa cells were grown in a fetal bovine serum/DMEM-F12-based medium. At approximately 50-60% confluency, cells were washed twice and transfected with a reporter vector and, where appropriate, the ER expression vector. Plasmids used were constituted as follows: vitellogenin A2 ERE was inserted into pCAT promoter reporter plasmid containing the early sv40 promoter. Both wild type and mutant mouse ER cDNAs were used for insertion into the pSG5 expression vector. After five hours, cells were washed and the appropriate test chemicals added. The effects of mouse EGF and oestrogen were investigated for cells transfected with either the parent reporter or the vitellogenin A2 reporter in the presence or absence of the mouse ER expression vector. After 18 hours, cells were harvested and two wells were pooled for each determination of chloramphenicol acetyltransferase (CAT) activity. This involved incubation with [¹⁴C]chloramphenicol, Tris and acetyl coenzyme, followed by autoradiography and liquid scintillation counting.

Markiewicz *et al.* (1993): The model system was generally similar to that of Littlefield *et al.* (1990). These studies were, however, conducted in a variant of the main Ishikawa cell line, known as Ishikawa-Var I, which does not show a proliferative response to oestrogens but which is sensitive to their stimulatory effect on alkaline phosphatase activity. Statistical analysis was by Student's paired t-test.

Endpoints/data interpretation

Holinka *et al.* (1989): Cell counts were achieved by counting from three dishes for each group using a haemocytometer under inverted light microscopy, in order to derive the following:

- **Proliferation of cells attached to substratum of mammalian culture dishes:** by counting cells on days 0, 2, 5, 8 and 11.
- **Exponential growth rate :** Cell counts on days 0, 1, 2 and 3; cell cycle length was calculated using the relationship:

$$t_2 = \ln 2/k$$

where t_2 is time to double cell numbers and k is the slope of $\ln n = f(t)$. Statistical significance was assessed on the regression lines fitting log cell numbers for each group on each day.

- **Colony-forming efficiency:** expressed as number of colonies greater than 50 μm formed/100 cells seeded.
- **Labelling index:** derived from fraction of labelled cells and cell cycle length.

- **Metaphase index:** estimated as product of fraction of cells in metaphase and length of the cell cycle.

It was found that both oestradiol and serum increased the proliferation of cells when attached to the substratum of mammalian culture dishes; effects were additive. In addition, during the exponential growth phase oestradiol significantly decreased the time to double the number of cells, from 38 to 29 hours. Oestradiol was found to double the numbers of colonies formed under the test conditions only if serum or charcoal-filtered serum was added; no effects were observed if only basal medium was used. Together the labelling and metaphase indices provided a basis for assessment of the mitogenic effects of oestradiol by derivation of the length of G_1 , using certain assumptions. The calculations indicated that the hormone was reducing the length of this cell phase.

Littlefield *et al.* (1990) and Markiewicz *et al.* (1993):

- **Alkaline phosphatase activity:** by measurement of coloration developed from p-nitrophenol by enzyme-linked immunosorbent assay plate reader.

In the Littlefield *et al.* study, cells were initially treated with 10^{-9} M oestradiol over six days and the alkaline phosphatase activity was measured at intervals. A slight increase in activity was apparent by the end of the first day; by three days it had increased more than 10-fold and by six days it was 20-fold. A three-day treatment period was selected as the standard assay point for subsequent work. Stimulation of alkaline phosphatase activity was also seen following treatment when treated with a range of natural (including 17 α -oestradiol, oestradiol -17-stearate, Δ^5 -androstenediol) or synthetic oestrogens (including moxestrol, 17 α -ethynyl oestradiol, oestradiol-17-valerate, 11 β -CH₃O-16 α -I-oestradiol, diethylstilboestrol and 16 β -iodoestradiol). Concentrations down to 10^{-14} M were detectable, depending on the activity of the chemical; the effect of oestradiol was detectable to 10^{-12} M. No effect of treatment was apparent for a range of corticosteroids, progestins and androgens. In addition, when the effects of cotreatment of 10^{-9} M oestradiol and three anti-oestrogens (tamoxifen, 4-hydroxytamoxifen and keoxifene) were studied, antagonism of the stimulatory action of oestradiol was observed in a dosage-related manner: anti-oestrogens alone did not have any appreciable effect on alkaline phosphatase activities. Δ^5 -androstenediol, an aromatase inhibitor, displayed very weak activity (0.03% relative to oestradiol).

Markiewicz *et al.* derived dose-response curves for the various phytoestrogens tested. Despite having different concentrations exerting a half-maximal effect (EC_{50}), the same level of alkaline phosphatase activity was induced as for oestradiol when tested simultaneously at concentrations that had been previously determined to be sufficient to elicit a maximal effect. This was considered to indicate that the complexes formed between the oestrogen receptor and oestradiol were functionally equivalent to those formed with the other chemicals. In addition, when coumestrol, genistein or daidzein were co-incubated with an anti-oestrogen, clear evidence of inhibition was found.

Ignar-Trowbridge *et al.* (1993):

- **CAT activity:** expressed as percentage conversion of radiolabelled chloramphenicol from unacetylated substrate to acetylated products per 10^6 harvested cells.

Both EGF and oestradiol were capable of stimulating trans-activation in cells expressing both the mouse ER and the vitellogenin ERE. Pre-treatment of cells with a specific ER antagonist was shown

to inhibit the CAT activity levels achieved, thus confirming that transcriptional activation of the ER was necessary for the effect.

YEAST SCREENS

In recent years transfection techniques have been developed which permit the controlled insertion of hormonal receptor structures from various animal species, including humans, into *Saccharomyces cerevisiae* yeast cells. Many such models have been developed because of their value at various levels of scientific investigation. Models have been investigated as potential screens for chemical-receptor interactions, in the belief that such interactions are likely to form the basis of the chemicals' hormonal activity. Because of the control possible over these systems, detailed mechanistic investigations of chemical-receptor interaction and receptor structure have also proved possible.

Several papers have reported on potential models for identification and quantitation of oestrogenic chemicals; a number are reviewed below. The work included in some of these papers addresses aspects beyond the scope of the current review document. In such cases, only those aspects relevant to the development, use or assessment of the model as a tool for oestrogenic screening are included.

Berry *et al.* (1990) investigated the agonistic activity of the anti-oestrogens tamoxifen and ICI-164,384 on various oestrogen reporter genes and vectors expressing truncated or chimeric human oestrogen receptors (hER) with one of two independent hER transcriptional activation functions (TAF-1 or TAF-2). The paper focused mainly upon work in the human HeLa cell and chicken embryo fibroblast (CEF) cell lines, but also included data relating to a *Saccharomyces cerevisiae* yeast cell model with an hER.

Method

Berry *et al.* (1990) used the yeast strain TGY14.1 to yield the pYERE1 series of transformants.

Yeast cells were transformed with pYERE1, pYERE1/HEGO, pYERE1/HE19G and pYERE1/HE15. The chimeric vectors thus expressed wild type Gly400hER (HEGO) or truncated hER mutants (HE19G, HE15) from the PGK promoter. HE19G was constructed by replacing the region of HE19 containing glycine 400 with the corresponding region of HEGO. The lacZ gene coding for β -glucosidase was under the control of an ER-dependent promoter which contained an ERE located upstream of the TATA box sequence of the GAL1 promoter.

After transfection, cells were grown to an optical density, OD₆₀₀, of approximately 0.6 units in a suitable medium. Oestradiol, the appropriate test material or vehicle (ethanol) alone were then added at a series of concentrations and the yeast cells harvested two hours later. β -glucosidase activity was assayed and expressed as fold-stimulation ($\pm 10\%$), taking the control (ethanol alone) value as 1.

Subsequent development

Wrenn and Katzenellenbogen (1993) investigated the structural requirements for recognising on and responding to ligands by the hER. They developed a series of point mutations in the hormone-binding domain (HBD) of the receptor and measured resultant changes in sensitivity to the oestrogen 17 β -oestradiol or the anti-oestrogen trans-hydroxytamoxifen. Particular attention was given to identifying residues in the HBD that were important for ligand binding and transactivation function of the receptor, and to elucidating mechanisms by which hERA discriminates between agonistic and antagonistic ligands.

A protease-deficient yeast strain, BJ2168 (obtained from a commercial source), was modified to produce the BJ-ECZ strain used in this study. The reporter construct used consisted of the *Escherichia coli* β -galactosidase gene, *lac-Z*, driven by a yeast CYC-1 promoter and two tandem copies of the consensus ERE sequence. hER was constitutively expressed from a multicopy plasmid, YEpER, which also contained oriY for replication in yeast and encoded TRP1 to complement the *trp1* defect in the parent yeast. The reporter was linearised with AatI to enhance integration into the defective *ura3* locus of yeast. Transformation was achieved by the lithium acetate method, with selection by growth on minimal medium lacking uracil. Integration was confirmed and copy number ascertained by Southern blot hybridisation. Hybridisation signals were quantified by β -scanning. An isolate containing nine tandem copies of the reporter construct integrated into the *ura3* locus was selected for use.

Responsiveness to exogenous chemicals was initially assessed using a plate culture technique. Aliquots of the culture were incubated on plates overnight in the presence of test material. Plates were then inverted over a glass dish of chloroform for 15 minutes to permeabilise the cells. Whatman 541 paper soaked in a solution of X-gal in dimethylformaldehyde, and allowed to damp dry, was placed on the colonies and incubated for one to three hours until a blue colouration developed. An alternative method was developed which involved liquid culture of the yeast with a soluble substrate to permit spectrophotometric measurement of β -galactosidase activity. Aliquots of the culture were transferred to glass tubes, mixed with test chemical, and incubated for four hours at 30°C with vigorous shaking (325-350 rpm). Cells were then collected by centrifugation and β -galactosidase activity determined for whole, permeabilised cells.

Kohno *et al.* (1994) studied on the effect of ligand structure on oestrogen-mediated transcription using oestradiol and the anti-oestrogens tamoxifen, hydroxytamoxifen, nafoxidine, ICI-164,384 and ICI-182,780. Using methodologies similar to those of Wrenn and Katzenellenbogen (1993), used the BJ2168 yeast strain. A plasmid (PG-MS) was based upon the murine oestrogen receptor cDNA clone, MOR 100, which was cloned into the Sal I site of the yeast expression vector PG-1. Use was again made of an ERE-regulated *lac-Z* reporter gene.

Yeast cells were grown in minimal media to a fixed optical density (as defined by spectrophotometry) before addition of the test chemical. After two hours, the yeast cells were harvested, made permeable by SDS-chloroform treatment, and β -galactosidase activity measured using o-nitrophenyl- β -galactoside as substrate. Further work (not discussed here) involved the use of other yeast strains having varying protease deficiencies to study the relationship between the stability of the ER and level of protease activity.

Klein *et al.* (1994): In order to investigate the possible role of oestrogens in the sex differences seen in epiphyseal development and the onset of puberty in humans, a recombinant yeast cell assay was used to compare oestrogen levels in prepubertal girls and boys. The system used a plasmid, YEPKB1, containing the CUP1 metallothionein promoter fused to human ER cDNA. The reporter plasmid used, YRPE2, contained two copies of the frog vitellogenin ERE upstream of the yeast iso-1-cytochrome c promoter, fused to the structural gene for β -galactosidase. These plasmids were introduced into the yeast strain BJ3505 and selected by tryptophan or uracil auxotrophy.

Cultures were stored deep-frozen until required. Approximately three to seven days before assay, the yeast was plated on to an agar-based selective medium. Plates were cultured at 30°C for 36 hours, then stored at 4°C. On the evening before assay, a single colony from the plate was used to inoculate 1-2 mL of selective media and incubated overnight at 30°C with agitation. At the start of the assay the optical density of the medium was standardised by dilution and the yeast was induced by copper sulfate. Aliquots were added to 12 glass tubes containing oestradiol extracted from blood samples obtained from the study subjects. After

incubation with shaking for eight hours, samples were transferred to radioimmunoassay tubes and centrifuged to pellet the cells. After re-suspension, cells were permeabilised by chloroform and cell density was assessed by light scattering using a microtitre plate reader. Colour formation was measured by serial use of the plate reader over six hours.

An intensive validation exercise was performed on the assay system which included assessment of specificity against oestriol, 17α -oestradiol, oestradiol 17-glucuronide, oestriol 3-sulfate, oestriol 3-glucuronide, oestriol 3-sulfate, oestrone glucuronide, oestrone 3-sulfate, testosterone, dexamethasone, cortisol, aldosterone, progesterone, diethylstilboestrol, ethynyl oestradiol and mestranol.

Maier *et al.* (1995): The authors studied the potential oestrogenicity of extracts from two dioecious plants, osage orange (*Maclura pomifera*) and mulberry (*Morus microphylla*). Individuals of these species produce unisexual flowers and the sexes were analysed separately. In addition, the effects of a number of other chemicals were examined; these included coumarin, genistein, kaempferol and quercetin, gibberellin A₃, IAA, NAA, BA and progesterone. The BJ3505 strain of *Saccharomyces cerevisiae* was used. This included an ER plasmid, YEPE10, comprising a ubiquitin (ub) gene fused to hER cDNA in the yeast 2 μ natural plasmid. The yeast also contained an oestrogen-responsive reporter plasmid, YRPE2, including the *cycI* promoter fused to the *E. coli* β -galactosidase *lacZ* gene.

Plant materials were extracted from homogenised leaves and inflorescences into ethanol and a supernatant prepared by centrifugation. Yeast cultures were grown overnight in minimal medium containing copper sulfate. This induced production of ERs, because the gene was under the control of a yeast metallothionein (CUP1) promoter. At a defined optical density, cultures were exposed to test extract and incubated for six to seven hours. Cells were then harvested, lysed and centrifuged at 13,000 g for 10 minutes at 4°C. The supernatant was analysed for protein content by the method of Bradford and β -galactosidase activity assessed by triplicate assay of subsamples with transcriptional-assay buffer, addition of o-nitrophenyl β -D galactopyranoside and, after 30 minutes, measurement of absorbency at 420 nm by spectrophotometer. The plant extracts were standardised against a series of dilutions of 17β -oestradiol and results were expressed as "oestrogen equivalents". In addition, cultures with or without copper sulfate alone, or with different amounts of ethanol, were run as "negative controls". Data were analysed by Student's t-test.

Routledge and Sumpter (1996) reported on investigations of representatives of all the major groups of surfactants and their primary degradation products (Table 11).

In addition, data for 17α -oestradiol, 17β -oestradiol sulfate, genistein, bisphenyl-A and op'-DDT. were presented.

The model comprised yeast cells (strain not identified) containing a stable hER DNA sequence within the main chromosome of the yeast. Expression plasmids carrying the reporter *lac-Z* gene for β -galactosidase were also inserted. The yeast cells were stored deep-frozen until required and were then cultured in appropriate medium for approximately 24 hours at 28°C on an orbital shaker until an absorbency at 640 nm of 1.0 was attained (samples were stored for a maximum of four months before replacement).

The assay medium was prepared by adding 0.5 mL of a chromogenic substrate, chlorophenol red- β -D-galactopyranoside (CPRG), to 50 mL of fresh growth medium (the chromogen was used to monitor β -galactosidase activity by absorbency measurement at 540 nm). Aliquots (10 μ L) of the appropriate concentration of test material were added to a 96-well, optically flat-bottomed microtitre plate and allowed to evaporate to dryness. 200 μ L of the assay medium seeded with the yeast culture was added to each well. At each assay, blanks containing assay medium alone and samples of a series of 17β -oestradiol

concentrations were used to produce a standard curve for quantification. Plates were sealed and incubated for three days before colour development was measured using a plate reader.

Connor *et al.* (1996): The potential oestrogenic activity of two commonly-used chloro-S-triazine-derived herbicides, atrazine and simazine, was assessed in a series of model systems. These included *in vivo* rodent assays (uterine weight, progesterone receptor binding activity and peroxidase activity), the MCF-7 cell line, and a transfected yeast strain containing hER cDNA. The PL3 strain of *Saccharomyces cerevisiae* was transformed with the high-copy 2 μ -parent vector Yep10 or Yep10 vector containing the hER cDNA (YEp10-HEGO) using the lithium acetate procedure. Transformations were selected on minimal media plates supplemented with amino acids.

Individual colonies were used for the test assays. Aliquots containing oestradiol or one of the herbicides were spotted onto plates, allowed to dry, and incubated at 30°C. Photographs were taken at 24-hour intervals over five days to assess colony growth. Statistical analysis of data was by Duncan's new multiple range test (ANOVA).

Arnold *et al.* (1996 a and b): In two papers, Arnold and co-workers described the development and use of a yeast oestrogen screening model to which they applied the acronym "YES". The model used the BJ2407 *Saccharomyces cerevisiae* strain, which was transformed with pSCW231 or pSCW231-hER and YRP2. The expression plasmid pSCW231-hER was created by ligating an EcoR1 fragment of the cDNA of the hER into pSCW231. The reporter plasmid YRPE2 contained two EREs linked to the lacZ gene. Transformants were selected over three days by tryptophan and uracil auxotrophy.

Table 11

Major surfactants and their primary degradation products (from Routledge and Sumpter, 1996)

Class	Surfactant group	Chemical tested
Anionic	Linear alkylbenzene sulfonates (LAS)	Petrolab 550 + degradation product (SPC) Siene
	Branched alkylbenzene sulfonate	ABS
	Alcohol sulfates (AS)	Sodium n-octyl sulfate Sodium n-nonyl sulfate
	Alcohol ether sulfates	Dobanol
	Secondary alkane sulfonates (SAS)	SAS
	Others	Linear- α -olefin sulfonate Sodium lignsulfonate
Non-ionic	Alcohol ethoxylates (AE)	Synperonic A3 Genapol C100
	Alkylphenol ethoxylates (APE)	NP12E0 + degradation products (4-OP, 4-NP, NP1EC, NP2EC, & NP2EO)
Cationic	Tallow amine derivatives	Di(hydrogenated tallow) dimethyl ammonium chloride
	Bezalkonium chloride	¹² C- ¹⁴ C-alkyldimethylbenzyl ammonium chloride [ed?]
Amphoteric	Betaines	Coconut amido betaine Cocobetaine
	Imidazolines	N-b-hydroxyethyl oleyl imidazoline

For the assay a single yeast colony was grown overnight, then diluted with fresh medium and incubated overnight in the presence of the test chemical, using dimethylsulfoxide (DMSO) as vehicle. β -galactosidase activity was assessed by centrifugation of the cells, followed by re-suspension and permeabilisation with chloroform and sodium dodecyl sulfate. After equilibration, the reaction was terminated, cell debris removed by centrifugation, and absorbance measured. Results were expressed as Miller units, given by the equation:

$$(A_{420}/(A_{600} \text{ of } 1/10 \text{ dilution of cells} \times \text{volume of culture} \times \text{length of incubation}) \times 1000)$$

In the first paper, the influence of blood proteins on bioavailability was assessed by culturing cells in the presence of human albumin, sex hormone-binding globulin, or whole human or alligator serum. Serum was also used following charcoal-stripping. The chemicals used included 17β -oestradiol, diethylstilboestrol, *o,p'*-DDT, octyl phenol, dexamethasone, testosterone and the anti-oestrogen ICI-164,384. In the second paper, effects of exposure to equimolar concentrations of a number of chemicals and the effect of combined application were assessed. Chemicals studied included endosulfan, dieldrin, toxaphene and chlordane.

Endpoints/data interpretation

Connor *et al.* (1996):

- **Colony formation:** colony development assessed visually for uracil-deficient media in presence of test material.

Other models:

- **β -galactosidase activity:** assessed by measuring the colour change in the presence of a suitable chromatographic substrate.

Berry *et al.* (1990): The authors interpreted the data on yeast cells transformed with pYERE1/HE19G as indicating that TAF-2 has very little if any transcriptional activity in yeast, when compared with TAF-1. Transcriptional activity for HE19G was not increased by use of recombinant pYERE3/HE19G which contained three consensus EREs in tandem. There was evidence for the agonistic activity of 4-hydroxytamoxifen. In contrast, ICI-164,384 neither stimulated transcription nor blocked oestradiol activity. It was suggested this might be attributable to lack of penetration of the yeast cell.

Wrenn and Katzenellenbogen (1993): ER levels were fairly high in this yeast model and certainly higher than in the MCF-7 cell line as determined by binding of tritiated oestradiol. The plate culture system was highly sensitive, with colonies turning medium blue at 10^{-10} M oestradiol and dark blue at above 5×10^{-10} M. However, the anti-oestrogens tested behaved as agonists (*e.g.* blue colouration was seen at 10^{-8} M trans-hydroxytamoxifen) and oestradiol-induced activity was not inhibited by either anti-oestrogen tested (even at a 10^4 -fold molar excess of the anti-oestrogen). For the liquid assay method, β -galactosidase activity varied from undetectable in untreated yeast to a maximal response when treated at 10^{-8} to 10^{-6} M oestradiol. A 50 times higher ligand concentration was needed for strong colour development by the liquid culture compared with the plate method; this was attributed in part to use of lower yeast concentrations and a shorter incubation period. For anti-oestrogens, very high levels were required to induce β -galactosidase activity and the response did not reach a plateau before growth-inhibiting levels were attained (*e.g.* using trans-hydroxytamoxifen maximum levels were only 20-25 % of that for the

oestradiol). As for the plate assay, neither anti-oestrogen showed inhibiting activity when yeast cells were stimulated with oestradiol.

Kohno *et al.* (1994): A maximum of 20 to 25-fold induction of β -galactosidase activity over the control level was reported after exposure of yeast containing the ER to 10 nM oestradiol for two hours. Similar inductions were recorded with tamoxifen or hydroxytamoxifen, with maximal effects at 1 μ M. These chemicals did not inhibit the response to oestradiol. Nafoxidine showed partial agonism at 0.1 μ M and partial antagonism to oestradiol at 0.5-10 μ M. ICI-164,384 also showed partial agonism with a dose-response that reached a plateau at 100-500 μ M; however, this chemical and ICI-182,780 showed no antagonism to oestradiol.

Klein *et al.* (1994): β -galactosidase activity measurements were converted to oestradiol equivalent units by linear interpolation from a standard curve for oestradiol. Levels were very low in the absence of oestradiol, but could be increased by up to 1000-fold. During assay validation, the method sensitivity (as defined by oestradiol concentration resulting in a β -galactosidase activity two standard deviations above the mean of 10 samples of charcoal-stripped plasma) was <0.02 pg/mL. Intra-assay variation, assessed by eight-fold assay of a single sample, was 15% at 2 pg/mL oestradiol and approximately 50% at 0.02 pg/mL. Inter-assay variation, estimated from assay of pooled samples on six days, was 13% at 2 pg/mL, rising to approximately 60% at 0.02 pg/mL. Recovery was assessed using radiolabelled oestradiol, and accuracy by calculation of correlation coefficient against a commercially available hormone radioimmunoassay kit: values were $93 \pm 17\%$ and 0.90, respectively. The model proved highly specific for oestradiol. Cross-reactivity was low with other chemicals: oestrone (0.3%); diethylstilboestrol (<3%); oestriol (0.03%); oestrogen metabolites (<2%); testosterone, aldosterone, progesterone and cortisol (<0.01%); 17α -oestradiol (9%) and mestranol (0.4%). In contrast, the result for ethynyl oestradiol was 100%.

The assay of prepubertal children detected a statistically significant difference between oestradiol equivalents in boys (0.08 ± 0.2 pg/mL) and girls (0.6 ± 0.6 pg/mL). The sensitivity of this assay was assessed to be at least 100-fold greater than previously available (*i.e.* non-yeast) test systems, but noted the unexpected specificity of the system for oestradiol. They offered a number of possible explanations, but were unable to positively identify the reason. Suggestions included intrinsic differences in ER/ERE complex activation, the ether extraction procedures, differences in metabolism of the chemicals by yeast during the incubation period, and differences in adsorption by non-receptor components.

Maier *et al.* (1995): A good dose-response curve for oestradiol was reported, with half-maximal activity at approximately 1 nM, differences in activity were demonstrated between extracts from male and female plants and seasonal variations in level were shown, with the highest levels occurring before and during flowering. The yeast assay also proved valuable in the identification of the activity chemicals during purification. Of the other chemicals tested, only genistein induced transcriptional activity of the reporter gene in the yeast system (activity levels 2.5 times lower than oestradiol), again stressing the specificity of the system.

Routledge and Sumpter (1996): On the basis of triplicate assays of oestradiol responsiveness, the authors reported the sensitivity and reproducibility of the system to be high over a concentration range of 1.5 to 3072 ng/L, with 3 ng/L of 17β -oestradiol producing a detectable colour change. The system was responsive to 17α -oestradiol and 17β -oestradiol sulfate, genistein, bisphenol-A and, to a lesser extent, *op'*-DDT. Testing of various surfactants at concentrations from 50 μ g/L to 100 mg/L showed that SPC, Synperonic A3, Genapol and the amphoteric, cationic and anionic chemicals were not oestrogenic. High

levels of Synperonic A3 and some of the cationic and anionic chemicals lysed the yeast cells. A dosage-related effect was, however, established for the degradation products of APEs.

Connor *et al.* (1996): It was shown that the yeast would not grow on plates lacking uracil except in the presence of an exogenous oestrogen, oestradiol. Growth could be detected after one day and continued throughout the test period. In contrast, neither herbicide elicited any growth, a finding that was generally in agreement with the other assay systems used to address potential ER interactions. Using other test models, effects were seen on progesterone binding and uterine peroxidase activity, which the authors interpreted as possibly arising through other pathways not involving the ER.

Arnold *et al.* (1996 a and b): In one paper, inclusion of hER was shown to be necessary for responsiveness to oestrogenic stimulation by 17β -oestradiol, diethylstilboestrol, *o,p'*-DDT and octyl phenol. Dexamethasone, testosterone and the anti-oestrogen ICI-164,384, however, were not stimulatory in either yeast cell type. Coincubation with blood proteins or serum (stripped using dextran-coated charcoal) showed significantly reduced or no activity in the presence of human albumin or SHBG, although the magnitude of effect depended on the chemical. Differences were also seen comparing whole human serum to alligator serum. The authors considered this further evidence for the multiplicity of factors involved in chemical oestrogenicity. Suggested factors included receptor binding affinity, availability in serum, persistence in the body, possibility for bioaccumulation, and the existence of species-specific serum protein compositions.

In the other paper (Arnold *et al.*, 1996a), exposure to dieldrin, endosulfan and toxafene only weakly increased the β -galactosidase activity. When given in combination, any two of these chemicals produced a synergistic effect. For example, the concentration needed for an endosulfan-dieldrin mixture was 1/160th to 1/1600th that required for either chemical alone. Chlordane, which was not in itself oestrogenic, also enhanced the potency of the other chemicals. Subsequent to the publication of this study, a number of other groups attempted to report these observations without success (Ramamoorthy *et al.*, 1997; Guidis *et al.*, 1997; Ashby *et al.*, 1997) and, in July 1997, the authors of the original paper officially withdrew their report as they were unable to replicate their own findings (McLachlan, 1997).

ANDROGEN-RELATED – YEAST SCREEN

In addition to the yeast models developed to identify chemicals capable of interacting with the oestrogen receptor (see Section 0) an androgen-inducible expression system based upon the yeast *Saccharomyces cerevisiae* has been reported by Purvis *et al.* (1991). The intention of the authors was to produce a controllable expression system based upon the human androgen receptor. The androgen, dihydrotestosterone (DHT), was used as the model chemical.

Method

Transformants of *S. cerevisiae* BJ1991 (α pep4-3, prbl-1122, ura3-52, leu2, trp1, GAL) were developed using standard conditions for all DNA digestions, ligations and transformations. Cells were grown in shaken liquid culture at 30°C. For GAL1.10 promoter induction, cells were pre-grown in SD medium, then transferred to YP-GAL medium for further incubation. *E. coli* strain TG1 [K-12, Δ (lac-pro), supE, thi, hsdD5[F⁻ traD36, proA⁺B⁺, lacI^q, lacZ Δ M15]] was used during plasmid construction. The constructs developed are summarised below:

Plasmid ^a	Parental plasmid	Replication element	Selection ^b	Induction ^c
pPGK-hAR	pMA91	2 μ	Leu	GLU \uparrow
pPGKgal-hAR	pKV49	2 μ	Leu	GAL \uparrow GLU \downarrow
pPGKare-hAR	YEplac181	2 μ	Leu	DHT \uparrow
pPGK-hARI	YIplac128	integrated	Trp	GLU \uparrow
pPGKare-lacZ	YEplac195	2 μ	Ura	DHT \uparrow
pPGKare-lacZC	YCp50	CEN4	Ura	DHT \uparrow
pPGKare-lacZI	YIp5	integrated	Ura	DHT \uparrow
pPGK-lacZ	pMA91	2 μ	Leu	GLU \uparrow
pPGKgal-lacZ	pKV49	2 μ	Leu	GAL \uparrow GLU \downarrow

^a Plasmid nomenclature was as follows: the basic promoter was denoted (*e.g.* PGK) followed by the UAS if different from the wt (either gal or are), then by the transcript (lacZ or hAR). I or C indicates the plasmid yeast replication method if not 2 μ .

^b The selection and maintenance of recombinants were achieved using either the URA3 or LEU2 prototrophic marker gene, because loss of the plasmid would result in inability to grow in the medium in the absence of either uracil or leucine, as appropriate.

^c Arrow indicates promotion or repression of transcription activity if grown on medium containing glucose (GLU) or galactose (GAL) as the sole carbon source or DHT.

Assessment of expression of the androgen receptor was by standard Western blot and *in vitro* band shift techniques, plus the ability to transactivate a hybrid PGK promoter carrying MMTV androgen response elements.

Endpoints/data interpretation

- **Western blot bands:** obtained by development of colour using a naphthol-containing solution.

- **Band shift assay:** looking for ability to bind double-stranded oligo linkers (67 bp) carrying copies of the ARE from the MMTV-LTR.

The successful development of a number of transformed cell types was reported, in which expression of the gene encoding the human androgen receptor from a strong yeast promoter resulted in transactivation of a hybrid promoter carrying androgen-responsive sequence. Western blot analysis showed that cells containing the pPGKgal-hAR plasmid produced an immuno-reactive protein of about 95 kDa in galactose grown cells. This did not occur in glucose-grown or host cells alone. This band also occurred for the 2 μ pPGK-hAR plasmid. No band shift in [³²P]-labelled oligos was seen in host cells alone or glucose-grown cells carrying the pPGKgal-hAR 2 μ plasmid, even in the presence of steroid ligand. However, significant retardation of the linker sequences was noted in galactose-grown cells carrying this plasmid. Addition of DHT did not have any apparent effect. The band shift was not seen where extracts from galactose-grown host cells were used alone. Labelled, dimeric ERE oligo, from the gene encoding *Xenopus laevis* vitellogenin, did not show retardation with respect to any extract, even when either DHT or oestradiol was added. Also, unlabelled ERE could not compete with ARE band shifting in extracts with hAR synthesis. On this basis, it was concluded that functional hAR was only present in cells carrying the expression system, after induction with galactose. Adjusting the combination of receptor, target-gene copy number and concentration of DHT permitted expression levels to be set within a 1400-fold range without apparent effect on normal cell growth.

**IN VIVO SYSTEMS RELEVANT TO ASSESSMENT OF EFFECTS ON WILDLIFE
(OESTROGEN RELATED)**

IN VIVO VITELLOGENIN INDUCTION ASSAYS

It has been recognised for a number of years that a normal part of egg development in the females of many egg-laying species involves the production of an egg protein, vitellogenin, by the liver in response to oestrogenic stimulation. This protein normally passes via the blood to the ovaries. The presence of this protein has been documented for female birds, reptiles, amphibia and fish. The vitellogenin is not generally detectable in immature animals or mature males, due to a lack of oestrogenic stimulation, but has been shown to be produced in response to exposure to exogenous oestrogenic stimulation. Evidence has accumulated that this protein may be a suitably sensitive marker for use in both laboratory and field-based *in vivo* studies, because methodology exists for its ready detection in blood samples. The potential significance of the production of this protein by males, and its use as a biomarker, has been extensively reviewed in a number of recent assessments and reports (*e.g.* Miljøprojekt nr. 290, Ministry of Environment and Energy, Denmark (1995); Assessment A1 MRC: Institute for Environment and Health (1995); Texte 3/36 Federal Environmental Agency, Germany (1996)). In order to demonstrate the range of methodologies that have been used, a number of papers studying various animal groups are briefly reviewed below.

METHOD

Birds

In a laboratory-based study, **Robinson and Gibbins (1983)** investigated a range of markers for vitellogenin induction in mature male Japanese quail following administration of either 17β -oestradiol, diethylstilboestrol, ethynyl oestradiol, methoxychlor, moxestrol, cholecalciferol, *o,p'*-DDT, testosterone, progesterone or zearalenone.

Male Japanese quail three to five months of age were housed under standard conditions and, on the first day of study, received an intramuscular injection of vehicle alone (negative control), oestradiol or diethylstilboestrol (depending on vehicle used; positive control), or one of the test materials. Injections were generally given as a single dose, except for the high dosage which was administered as two equal doses, two hours apart. Blood samples were taken pre-treatment, and one, two, four, six and eight days after administration and analysed using established methods for plasma protein-bound phosphorus, total calcium and packed cell volume (PCV), as these were considered to be markers for vitellogenin induction. Blood samples taken six hours after dosing were rocket immunoelectrophoresed, using antibodies for quail vitellogenin. These samples were diluted with Tris-HCL containing phenylmethylsulfonyl fluoride and sodium chloride. Glass plates subbed with an agarose and water mix were covered by a solution containing agarose and antibody was added. Antigens were placed in wells situated along one edge of the plate and electrophoresis was conducted for 20 hours. After washing and staining with Coomassie brilliant blue, the plates were photographed. Plasma samples taken after four days of treatment were electrophoresed using a discontinuous SDS-polyacrylamide gel method, followed by overnight fixation in a methanol-acetic acid-water mixture and staining Coomassie brilliant blue, to show the presence of the protein subunits of vitellogenin.

Amphibia and reptiles

Palmer and Palmer (1995) reported on the detection of vitellogenin induction in adult male red-eared turtles (*Trachemys scripta*) and African clawed frogs (*Xenopus laevis*) by xenobiotic oestrogens and suggested its use as a suitable biomarker in reptiles and amphibians. Animals were obtained from commercial sources and housed under standard husbandry conditions. Female turtles were treated with oestradiol and sampled to obtain vitellogenin, which, following purification and characterisation, was used for antibody production in rabbits.

Male turtles were assigned to treatment groups and received the vehicle alone, or the appropriate treatment by intraperitoneal injection daily for seven days. Adult male frogs were subject to a similar treatment regimen. Chemicals investigated included 17β -oestradiol, diethylstilboestrol and *o,p'*-DDT. Blood was collected from the caudal vein of each animal on study day 14 and the plasma obtained by centrifugE. This was then deep-frozen until use. Relative levels of vitellogenin in plasma were assessed using an enzyme-linked immunosorbant assay (ELISA) technique. ID-SDS-PAGE and Western blot analyses were also performed.

For the ELISA assay, plasma samples with excess bovine serum albumin were diluted with TBS and Tris and incubated overnight at 4°C. After washing, plates were blocked with Blotto-Tween for two hours, then re-washed and incubated with anti-rabbit immunoglobulin conjugated to alkaline phosphatase. After washing, the plates were developed with p-nitrophenyl phosphate and incubated for 15 minutes at room temperature before the reaction was stopped by addition of Tris and EDTA. Plate densities were read using an automated reader. In addition, where appropriate, whole plasma was solubilised in Tris buffer containing sodium dodecyl sulfate and 2-mercaptoethanol and separated by SDS polyacrylamide gel electrophoresis (ID-SDS-PAGE). Separation gels were made from acrylamide. Gels were fixed and stained with Coomassie blue and silver and molecular weights determined against BioRad high molecular weight standards using calculation of R_f values. Vitellogenin was quantified from the ID-SDS-PAGE using an imaging densitometer. Immunological identification of vitellogenin was achieved using a Western bolt technique. This involved transfer of the plasma samples to polyvinylidene difluoride membranes immediately following ID-SDS-PAGE. A 15-volt electrical field was applied for one hour. After transfer, the membrane was equilibrated in Tris-buffered saline for 15 minutes, blocked with 5% w/v powdered milk in TBS for two hours, and incubated overnight with primary antibodies specific for vitellogenin. The transfer membrane was then washed and incubated with goat anti-rabbit immunoglobulin conjugated to alkaline phosphatase. After further washing, the membrane was incubated with nitro blue tetrazolium, bromo-4-chloro-3-indoyl phosphate and magnesium chloride to localise the reactive proteins.

Data were analysed by Kruskal-Wallis one-way analysis of variance on ranks (ANOVA) and the Student-Newman-Keuls method.

Fish

Tyler and Sumpter (1990) reported on the development of a radioimmunoassay (RIA) method to detect vitellogenin in the mirror carp *Cyprinus carpio* and the testing of its reactivity in a range of other carp species.

RIA development: Vitellogenin (c-VTG) was induced in two year old mirror carp by intraperitoneal injection of 17β -oestradiol and resolved from other blood components using a combination of gel-filtration on Sepharose 6B and ion-exchange on DEAE-cellulose in the presence of the enzyme inhibitor aprotinin. The concentration of c-VTG standard used in this study was determined on the basis of a pre-

calibration ratio using optical density at 276 nm of the c-VTG and subsequent determination of sample protein weight by freeze-drying. Antibodies were raised in half-lop rabbits by repeated intracutaneous injection of c-VTG in Freund's complete adjuvant. Blood was collected and serum separated. The c-VTG was found to be highly immunogenic in all rabbits used. The antibody chosen for the RIA was used at a final concentration of 1:90,000, at which dilution approximately 50% of the label bound in the absence of unlabelled VTG. Antibodies for rainbow trout (rt-VTG) were supplied by other workers for the comparative aspects of this study. Samples of VTG were radiolabelled with Na¹²⁵I using Iodogen as the oxidising agent. Labelled VTG was separated from free iodine by gel-filtration on PD10 columns containing Sephadex G25 pre-saturated with bovine serum albumin. Labels were stored deep-frozen and checks on stability performed over a two-month period. Cross-reactivity of these two antibodies was assessed by incubation of serial dilutions with the heterologous label.

Blood sample collection: Mirror carp at various stages of sexual maturity and two other subspecies of *Cyprinus carpio*, the common and Koi, and the grass carp, *Ctenopharyngodon idella*, were obtained from commercial sources and blood samples were taken under anaesthesia. Samples were similarly obtained from wild caught crucian carp, *Carassius carassius*, tench, *Tinca tinca*, roach, *Rutilus rutilus*, bream, *Abramis brama*, and dace, *Leuciscus leuciscus*. Blood was also taken from commercial or laboratory-sourced rainbow trout, *Salmo gairdneri*, brown trout, *Salmo trutta*, and chinook salmon, *Oncorhynchus tshawytscha*. Samples were either used immediately or stored deep-frozen until assay.

Assay procedure: Samples (50 µL) of standard c-VTG or unknown were added to similarly sized samples of antibody and radiolabelled c-VTG, vortexed, and incubated for five to six hours at room temperature. Separation of bound from free VTG was achieved by overnight incubation with anti-rabbit γ -globulin, followed by centrifugation. After aspiration of the supernatant, precipitated, labelled VTG was measured by scintillation counter. All assays were run in duplicate. In order to validate the assay, blood samples were analysed from mirror carp of both sexes, of varying stages of sexual development, and analysed. After blood sampling, fish were killed and the gonadosomatic index calculated to provide an index of stage of sexual development. In further experiments, plasma calcium was measured using a proprietary chemical analyser for a range of species and ages of fish.

Subsequent development

Pelissero et al. (1991) reported on the experimental induction of vitellogenin in the Siberian sturgeon, *Acipenser baeri*, following intraperitoneal injection with a phytoestrogens (Formononetin, daidzein, equol, biochanin A, genistein or coumestrol) and comparison of the response with that of 17 β -oestradiol-treated animals. Blood was collected before treatment from ten randomly chosen animals for baseline analysis. Groups of yearling fish (four per group) received the relevant treatment on alternate days over an eight-day treatment period; blood was taken for analysis on day 10 of the study. The system used for assay of plasma vitellogenin in this study was a homologous enzyme linkage immuno-sorbent assay (ELISA) using sturgeon-specific immunoserum raised in the rabbit.

Kishida et al. (1992) collected blood and mucus samples from the body surface of wild caught striped bass, *Morone saxatilis*, taken from the Hudson River in the United States. Gonads from the animals were retained deep-frozen. The effects of oestradiol administration were also studied on captive striped bass.

Vitellogenin levels were measured in plasma, mucus and ovarian extracts using an ELISA method involving goat- or rabbit-derived antibodies to striped bass vitellogenin. The putative vitellogenin protein induced in the oestrogen-treated animals was further characterised by isolation using SDS-polyacrylamide gel electrophoresis (SDS-PAGE), followed by application of a proprietary Western blot assay kit.

Periera *et al.* (1992): As part of a larger investigation, these authors studied vitellogenin expression in the winter flounder, *Pleuronectes americanus*, wild caught in Long Island Sound or Boston Harbour. In this study, fish were caught from a series of stations in relatively clean or degraded areas at monthly intervals. They were then held in running sea water overnight and a blood sample was taken. Samples were refrigerated overnight to permit clot formation, centrifuged, and the serum stored deep-frozen until analysis. In this experiment, vitellogenin was not measured directly but was inferred from the presence of alkali-labile phosphate in the serum using a modification of the Wallace and Jared method (1968). Data were subject to Shapiro-Wilk analysis to assess normality, followed by two-way analysis of variance and Duncan's multiple range test.

Purdom *et al.* (1994) investigated the oestrogenic effects of sewage effluent water on fish by housing rainbow trout in cages in or near effluent discharges. In addition, the potency of constituents of the contraceptive pill was assessed under laboratory tests. Immature trout were obtained from commercial sources and acclimatised before study.

For field exposures, animals were housed in galvanised steel cages and not fed during the test period. The following regimens were studied:

- Preliminary study: Trout were held at the main sewage treatment works (STW), nearby at a smaller STW, at a Ministry of Agriculture, Fisheries and Food (UK) experimental station below a small STW, at a tap water supply site, and at a spring water supply site (the latter two sites constituting controls).
- Preliminary study: Trout held at main STW site for varying times.
- Main trial: At 30 main STW sites throughout England and Wales both trout and carp were investigated.
- In addition, there was a series of trials of linked STWs, potable water intake points and intermediate sites using trout.

Laboratory studies were also conducted in which male trout received intramuscular administration of ethynyl oestradiol or 17 β -oestradiol. Subsequently, immature carp or male trout were held in aquaria with through-flow water, into which chemicals were fed by peristaltic pump at 25 ng/L. Chemicals studied included ethynyl oestradiol, mestranol and the α - and β -glucuronides. Samples were taken pre-treatment and daily for six days.

Blood samples were obtained under anaesthesia. At humane killing, length and sex of animals were recorded. Plasma was retained in liquid nitrogen until analysis by a trout RIA for vitellogenin. The RIA described above (Tyler and Sumpter, 1990) was used for the studies on carp; assays were performed blind on coded samples.

Sheahan *et al.* (1994) presented the results of further work on the effects of oestrogenic stimulation on rainbow trout, in which the effects of temperature upon response was investigated. Individually identified one-year-old fish were held in groups of 16 in aquaria at either $11.4 \pm 0.3^\circ\text{C}$ or $17.4 \pm 1.1^\circ\text{C}$. Animals were exposed to one of three concentrations of 17 α -oestradiol, via the water, for 28 weeks. A control (unexposed group) was also included. As part of a series of measurements, blood was collected after two, 12, 20 and 28 weeks during the exposure period and the plasma obtained was stored deep-frozen until

analysis for vitellogenin by immunoassay using a homologous rainbow trout antibody. Data were logarithmically transformed and subjected to one-way ANOVA. Vitellogenin was also compared in relation to temperature, maturity stage, and exposure concentration using a general linear model.

Ren *et al.* (1996) reported on the development of a cDNA probe to detect the induction of vitellogenin in rainbow trout liver by DDE, nonylphenol or 17 β -oestradiol.

In developing the probe, two primers for the polymerase chain reaction (PCR) were synthesised by and reverse transcriptase PCR was performed using mRNA and primers for 40 cycles. A 1.2% agarose gel slice of 1100 base pair cDNA (Vg 1.1) was cut out from the slow-melting agarose gel for further subcloning. A 4.1 kb vector pSG5 was prepared for subcloning by digestion with BamHI from a commercial source and treatment with alkaline phosphatase. This was followed by phenol extraction and ethanol precipitation. One pM of phosphorylated Vg 1.1 cDNA and five of dephosphorylated pSG5 were ligated with T4 DNA-ligase (from a commercial source) overnight and transformed into competent AG1 *E. coli* host cells and grown on plates. Colonies were selected and grown in culture medium, and plasmid preparation was accomplished using a kit from a commercial source. The pSG5Vg1.1 plasmid formed was labelled for use in Northern blot analysis, using a commercial kit, and stored deep-frozen until use. Storage over several months was possible in these conditions.

Sexually immature male and female rainbow trout (*Oncorhynchus mykiss*) were obtained from commercial sources and held under standard conditions in a through-flow system. Groups of three trout received nonylphenol via the through-flow water for three days, or single intraperitoneal injections of vehicle alone, or solutions of DDE or 17 β -oestradiol.

RNA was purified from the livers of fish using Trizol reagent followed by electrophoresis on an agarose-based gel, incubation with sodium hydroxide, and transference to a nylon membrane. RNA was immobilised using a UV-cross linker and the blot pre-hybridised in a sealed plastic bag with hybridisation buffer; 50 ng of labelled probe was added to the hybridisation buffer and the membrane incubated for 16 hours at 60°C. After washing, the membrane was placed in liquid blocking agent in diluent buffer and agitated for one hour. After further washing, the membrane was sprayed with diacetone detection reagent and placed in a film cassette for a two-hour exposure.

Nimrod and Benson (1996) reported on an investigation of the oestrogenic responsiveness of the Channel catfish, *Ictalurus punctatus*, using vitellogenin induction as one of the endpoints. Juvenile catfish received single intraperitoneal injections of and compared with that for the striped bass. From this, a consensus peptide 15 amino acids long was assembled using an automated peptide synthesiser and purified by reverse-phase high-performance liquid chromatography. Aliquot of the purified peptide was conjugated to maleimide-activated carrier proteins, keyhole limpet haemocyanin or ovalbumin and used to immunise rabbits. Serum containing the antibodies was stored deep-frozen until use. Purified vitellogenins and serum from striped bass were separated by SDS-PAGE and Western blots and used to test the immunoreactivity of the antibodies thus developed.

UNIVERSAL VITELLOGENIN ASSAY

Heppell *et al.* (1995) reported on the state of development of assay systems for vitellogenin monitoring of a wide variety of species. In many ways the immunological and structural properties of vitellogenin can vary greatly, even among closely-related species, but there may be some highly conserved regions that could be exploited to develop antibodies and immunoassays of a more universal nature.

Balb/C mice were immunised by repeated exposure to vitellogenin from either rainbow trout (*Oncorhynchus mykiss*) or striped bass (*Morone saxatilis*) in the presence of Freund's complete adjuvant and monoclonal antibodies (mAbs), generated using standard methods. Hybridoma colonies were screened for cross-reactivity using an antibody capture ELISA assay against purified vitellogenins, blood plasma from uninduced immature or male striped bass, or trout or animals injected with 17 β -oestradiol. The mAbs were harvested by collection of the tissue culture supernatant (TCSN).

The TCSN was mixed with goat serum and added to a 96-well plate coated with antigen. The second antibody was a goat anti-mouse IgM-specific antibody, conjugated with horseradish peroxidase. Colonies whose TCSN showed immunoreactivity to vitellogenin in the ELISA and to plasma from oestradiol-treated animals of both species were selected for cloning. The mAbs from the monoclonal cultures were isotyped using commercial kits. In addition, Western blots were conducted as follows. Samples of vitellogenin and plasma from oestradiol-treated animals were separated by sodium dodecyl sulfate electrophoresis (SDS-PAGE) and electroblotted onto membranes. The membranes were blocked overnight with buffered saline and bovine serum albumin, incubated with TCSN diluant for two hours, and then probed with secondary antibody. Binding was visualised with diaminobenzidine. ELISA and Western blot screens were also performed on the mAbs, using vitellogenin-containing plasma as well as plasma not containing vitellogenin from a variety of species such as the white perch (*Morone americana*), white croaker (*Genyonemus lineatus*), brown trout (*Salmo trutta*), pollock (*Theragra chalcogramma*), tuatara (*Sphenodon punctatus*), rattlesnake (*Crotalus molossus*) and chicken (*Gallus domesticus*). Additional species investigated using the Western blot technique included the bullfrog (*Rana catesbeiana*) and the black rat snake (*Elaphe obsoleta*).

The amino acid sequence of vitellogenin from a number of species of teleost fish was derived and a consensus N-terminal sequence analysed with a protein structure programme. From this, a consensus peptide of 15 amino acids was produced using an automated peptide synthesiser. An additional cysteine residue was added to enable N-terminal for coupling the peptide to activated carrier proteins. The peptide was cleaved from the resin with trifluoroacetic acid, purified by reverse-phase high performance liquid chromatography, and then conjugated to maleimide-activated carrier protein, keyhole limpet haemocyanin or ovalbumin. The keyhole limpet haemocyanin conjugate was used, in conjunction with Freund's complete adjuvant, to immunise New Zealand white rabbits. Serum obtained from the rabbits was stored deep-frozen until use. Purified vitellogenin and serum from striped bass were separated by SDS-PAGE and Western blot and used to test the immunoreactivity of the antisera to the vitellogenins and other serum proteins. Western blot visualisation was by goat anti-rabbit IgG, conjugated to alkaline phosphatase as the secondary antibody.

ENDPOINTS/DATA INTERPRETATION

Robinson and Gibbins (1983):

- **Blood protein-bound phosphorus, total calcium and packed cell volume:** using established methodology.
- **Vitellogenin:** visualised by either SDS-polyacrylamide gel electrophoresis or rocket immunoelectrophoresis, using antibodies for quail vitellogenin.

The authors recorded a gradual increase in plasma protein-bound phosphorus, total calcium and total protein and a decrease in PCV from day 4 to 8 only after treatment with the known oestrogens or zearalenone. The results of SDS-polyacrylamide gel electrophoresis similarly showed the presence of the vitellogenin subunits in samples from animals given these treatments. After only six hours, rocket immunoelectrophoresis allowed detection and quantitation of increased expression of vitellogenin in these groups, underlining the power of this antibody-mediated tool.

Tyler and Sumpter (1990); Pelissero *et al.* (1991); Kishida *et al.* (1992); Purdom *et al.* (1994); Sheahan *et al.* (1994); Palmer and Palmer (1995); Nimrod and Benson (1996):

- **Vitellogenin:** using radiolabelled vitellogenin and RIA or ELISA methodology. (Palmer and Palmer also used ID-SDS-PAGE or Western blot analysis).
- **Plasma total calcium:** by chemical analyser (Tyler and Sumpter only).
- **Hepatic-somatic index (HSI):** calculated from bodyweight and liver weight (Nimrod and Benson only).

Tyler and Sumpter showed the radiolabelled c-VTG to be stable over a period of 60 days. The routine radioimmunoassay procedure adopted permitted detection of c-VTG at 2-200 ng/mL and was very reproducible. The assay was run in a relatively insensitive manner, because comparatively high levels of VTG were detectable even in juvenile females using this system and therefore dilution of samples was adopted as necessary. Levels of mature female carp increased by more than 1000 fold, whilst plasma from the males had non-detectable levels in all cases (<2 ng/mL). Similar responses were found in the case of common, grass and Koi carp. Minor differences in response curve were, however, seen in the crucian carp. Similarly, the responses in respect of roach, dace and bream paralleled the c-VTG standard. Response in respect of tench was less accurate. Comparison of binding affinities for antibodies and VTG from mirror carp and rainbow trout showed that c-VTG did not bind well to the trout antibodies and vice versa. This was reflected in the very weak cross-reactivity found using the c-VTG RIA on the various salmonid species. Results from plasma calcium measurements showed a varying correlation with vitellogenin concentrations, depending on species and stage of development. The authors therefore considered this method to be of questionable general application.

The ELISA method used by Pelissero *et al.* was reported to have a lower limit of sensitivity of 0.1 µg vitellogenin/mL plasma. All the phytoestrogens tested, with the exception of formononetin, induced vitellogenin formation in the sturgeon, although to a lesser extent (on a dosage-related basis) than oestradiol.

Kishida *et al.* demonstrated that the ELISA method permitted satisfactory quantification of the vitellogenin, with serially diluted plasma from female striped bass showing parallelism with the standard curve. Assay sensitivity was shown to be high, with a limit of detection equivalent to a plasma concentration of 160 ng/mL. Dilution of some samples was necessary, because recoveries of vitellogenin from plasma or mucus were unreliable at high concentrations. The ELISA method was

similarly shown to detect vitellogenin satisfactorily in ovarian extracts and body mucus. Recovery in plasma and mucus was shown to be 113 and 100%, respectively, whilst intra- and inter-assay variation was 13 and 14%, respectively, at approximately 50% binding. This study confirmed the expected picture, with the synthesis of vitellogenin in both male and female fish treated with oestradiol. Vitellogenin was present naturally in mature females, with levels of 114-558 µg/mL in females which had not yet spawned. A positive response was not found for untreated males. SDS-PAGE showed the presence of a specific peak in the oestradiol-treated as compared to the untreated fish, which corresponded to a 170 kDa protein. Further investigations, involving sample passage over a Bio-gel A-1.5m column and comparison against known molecular weight chemicals, suggested that the native vitellogenin protein probably existed as a dimer of 170 kDa subunits. After concentration by ultracentrifugation and Western blot analysis, the specificity of the relationship between the induced protein fraction and the vitellogenin antibody used in the assay was confirmed.

Purdom *et al.* demonstrated that exposure to sewage outflow resulted in a rapid elevation of plasma vitellogenin levels (>1000-fold in three weeks; maximum elevation found at any site was 100,000-fold). They also demonstrated that induction levels were less in carp than in trout. The laboratory work further found that levels as low as 1 to 10 ng/L of ethynyl oestradiol would elicit a response. Indeed, the assay methods were sufficiently sensitive to give a positive response at 0.1-0.5 ng/L. Sheahan *et al.* observed a general increase in vitellogenin levels throughout the treatment period in rainbow trout exposed to 17α-oestradiol, although difficulties in the identification of individual fish because of tag loss limited analysis of data. Statistically significant increases were principally apparent in males given 1 ng/L at both temperatures and males given 0.3 ng/L held at the lowest temperature.

Palmer and Palmer demonstrated significant induction of vitellogenin in turtles following administration of oestradiol, diethylstilboestrol or DDT; the effect of DDT was less than that of the other two chemicals. No vitellogenin was extractable from control plasma or detectable using ID-SDS-PAGE or Western blot analysis, indicating that a slight absorbance noted for control plasma in the ELISA analysis was attributable to non-specific binding of polyclonal antibody. In the frog, a protein of approximately 200 kDa was extractable from plasma taken from treated but not control animals, but no cross-reactivity was found using the anti-turtle antibody. However, this was identified as vitellogenin using ID-SDS-PAGE or Western blot analysis and showed a similar treatment- and dose-dependent pattern of response to that seen in the turtle.

Nimrod and Benson reported that in the case of induction of vitellogenin production by ethynyl oestradiol, mestranol, diethylstilboestrol and p-nonylphenol, they were able to quantitatively compare potencies with the positive control, 17β-oestradiol. The anti-oestrogenic action of tamoxifen was apparent when co-administered with oestradiol. No response was observed for chlordecone, lindane, β-hexachlorocyclohexane and op'-DDT. Induction of vitellogenin was a more sensitive marker than HSI. For example, oestradiol increased vitellogenin from a dosage of 0.01 to 0.1 mg/kg, yet increase in HSI was not observed until between 1 and 10 mg/kg. In addition, they commented that the liver of the fish may increase in size through other mechanisms, *e.g.* metabolically induced hypertrophy. A potential problem was reported on the use of vitellogenin induction as a biomarker for oestrogenicity or anti-oestrogenicity: this was the variation in responsiveness between individuals, the cause of which was not resolved in this study.

Periera *et al.* (1992):

- **Serum alkali-labile phosphate:** using a modification of the Wallace and Jared method (1968).

There were some differences between the sites sampled, which corresponded to the size of fish considered. Thus elevated alkali-labelled phosphate levels were recorded for large, but not small, fish caught in the degraded areas compared with the clean areas.

Ren *et al.* (1996):

- **Vitellogenin mRNA induction:** detected using a cDNA probe in a pSG5 plasmid, using Northern blot analysis.

Treatment of fish with the test chemicals at dosages that did not elicit any overt toxicity nonetheless resulted in easily detectable induction of vitellogenin mRNA.

Heppell *et al.* (1995):

- **Vitellogenin antibody responsiveness:** measured using ELISA and Western blot techniques.

Seven mAB cultures were developed. The first type (R1) was an IgM antibody with strong cross-reactivity to vitellogenin from striped bass, rainbow trout, and the other species investigated. Regrettably, this line was lost due to equipment malfunction. The second line rapidly lost responsiveness, whilst the third culture, produced from mice immunised with bass vitellogenin, gave five types of IgM antibody, the most consist of which was named "2D8". This was believed to be responsive to trout, bass bull frog, black rat snake and chicken vitellogenin. In addition, initial investigations of the antiserum generated against the consensus N-terminal vitellogenin sequence produced from this series of experiments were reported to indicate a wide cross-reactivity with vitellogenins from a range of species. Further work on this aspect was in progress at the time of publication. In summary, the development of both monoclonal antibodies and polyclonal antisera possessing reactivity to vitellogenin derived from several species. The authors proposed the use of these tools as the basis for a universal immunoassay to detect oestrogenic exposure in any oviparous vertebrate, whilst recognising the need for further research to produce higher affinity mAbs and more specific antisera.

IN VIVO FISH ZONA RADIATA PROTEIN INDUCTION ASSAYS

There is some evidence suggesting that an alternative marker to oestrogen exposure, *zona radiata* protein expression, may be more sensitive than vitellogenin induction, although the literature is as yet sparse.

METHOD

Arukwe *et al.* (1997) conducted two experiments. In the first, juvenile Atlantic salmon, *Salmo salar*, approximately one year of age were maintained in continuously running sea water under natural daylight. Six groups, each containing six fish, were given single intraperitoneal injections of nonylphenol, oestradiol or vehicle alone. Blood samples were collected from the caudal vessel after 14 days and the plasma samples retained. Animals were fasted during this period. In the second experiment, similar fish were held in tanks containing high-quality drinking water containing effluent from an oil refinery treatment works at a range of concentrations. After four weeks, during which animals were fed every second day, plasma samples were again obtained.

In each case, the plasma samples were subject to immunochemical analysis using Western blotting and indirect ELISA techniques. ELISA involved the use of rabbit-derived polyclonal anti-salmon antibodies. For comparison, analysis of vitellogenin induction was also undertaken.

ENDPOINTS/DATA INTERPRETATION

- **Plasma vitellogenin and *zona radiata* protein levels:** by Western blot and ELISA analyses.

For the first experiment, Western blotting showed a cross-reacting vitellogenin protein. Three forms of *zona radiata* proteins (Zrp-alpha, Zrp-beta and Zrp-gamma) were detected in salmon exposed to the highest dose (125 mg/kg) of nonylphenol. At lower doses, only Zrp-beta showed any response. ELISA assay found immunoreactive vitellogenin and ZRP proteins at a dose of 125 mg/kg and Zrp only at 5 mg/kg. In the second experiment, effects were apparent for all parameters at the highest concentration (50% v/v effluent) but only Zrp-beta was affected at 1 or 10 % effluent.

***IN VITRO SYSTEMS RELEVANT TO ASSESSMENTS OF EFFECTS ON WILDLIFE
(OESTROGEN RELATED)***

IN VITRO VITELLOGENIN INDUCTION

In addition to the *in vivo* models described in Section 0, a number of authors have recently reported on the possibility of detecting xenobiotics' oestrogenic actions using *in vitro* test models of vitellogenin induction.

Pelissero *et al.* (1993) discussed the problems associated with *in vivo* and *in vitro* model systems. They pointed out that most of the metabolic processes that occur in the *in vivo* situation are normally absent from *in vitro* models, necessitating caution when extrapolating from *in vitro* to the whole animal. Nonetheless, they believed that an *in vitro* system that was capable of at least some of the metabolic transformations occurring *in vivo* would be useful for studying chemicals that require metabolic transformation before exerting oestrogenic activity. They therefore based their model upon a primary cell culture: the *in vitro* incubation of liver cells from the rainbow trout, *Oncorhynchus mykiss*. Further studies using similar methodology are discussed in Section 0.

METHOD

Rainbow trout were obtained from commercial sources. Fish were killed by a blow to the head and heparin was injected into the heart. The liver was then perfused through the intestinal artery, initially with saline and then with Hepe's buffered solution. After blood was removed from the liver, the perfusion was maintained for a further 30 minutes with Hepe's solution containing collagenase. The liver was then dissected and a cell suspension prepared. After repeated centrifugation and re-suspension with fresh medium, a concentrated suspension was prepared (10^6 cells/mL). Cell viability was assessed by Trypan blue exposure. Cells were decanted into 30 mm diameter wells (3×10^6 cells/well) and gently shaken continuously throughout culture. The medium was replenished at regular intervals during the period taken for the cells to aggregate. Once this was completed, cells were stimulated by addition of the appropriate test hormone; chemicals investigated included oestradiol, diethylstilboestrol, 17β -oestradiol glucuronide, testosterone, 11-ketotestosterone, progesterone and cortisol. The inhibitory action of tamoxifen was also studied. Effects of a series of phytoestrogens - including biochanin A, coumestrol, daidzein, equol, formononetin and genistein - were also investigated. Vehicle controls were used, as appropriate, and experiments were run in at least duplicate. For the initial experiment, the vitellogenin concentration of the medium was determined at intervals during incubation. Thereafter, a standard exposure period was used. Statistical analysis was by covariant analysis of regression lines derived from the vitellogenin dose-response data for each test material.

Livers from males, immature females or sterile animals were used. The observed dose-response curves were not significantly different. However, the data presented in the paper related solely to livers from immature females.

SUBSEQUENT DEVELOPMENT

Jobling and Sumpter (1993) reported on the use of a very similar test model to investigate the activities of a number of alkylphenol polyethoxylates (APEs). Significant differences in methodology from that of Pelissero *et al.* (1993) were restricted to the use of only hepatocytes from male (generally immature) fish and exposure of cells to the test chemical for a standard period of only two days. Multiple assays were again conducted for each set of experimental conditions. Statistical analysis was by non-linear regression and calculation of ED_{50} . These methods were used to calculate dose-dependent, oestradiol-relative

potencies. One-way analysis of covariance, followed by Scheffé's test for multiple comparisons, were then applied.

White *et al.* (1994b) presented further work on the effects of APEs using this test model.

Sumpter and Jobling (1995) reviewed the current state of knowledge relating to the use of vitellogenesis as a biomarker for oestrogenic contamination in water. In this paper, reference is made to little or no vitellogenin being expressed in immature and male fish. This is attributed to the circulating levels of oestrogens being too low to trigger gene expression.

ENDPOINTS/DATA INTERPRETATION

- **Vitellogenin concentration:** measured by radioimmunoassay. The dose-response curve (where sigmoid) for each test material was linearised and regression equations obtained. The data were compared to the system's response to oestradiol. Differences in sensitivity were seen between different cultures; it was therefore necessary to generate an oestradiol response curve for each experiment. The authors were not able to identify the mechanism responsible for this variation in hepatocyte sensitivity.

In the paper by Pelissero *et al.* (1993), during the initial investigation of oestradiol sensitivity vitellogenin production was assessed after 2, 4, 6 and 8 days' exposure. Maximal response was achieved by day 6, and this was subsequently used as the standard exposure duration. For oestradiol, minimal detectable stimulation occurred at 10 nM whilst 1000 nM was needed for maximal response. The test system was found not to express vitellogenin in the absence of xenobiotic stimulation.

In subsequent experiments, androgens and progestogens were found to induce vitellogenin although their potencies were approximately 1000 times less than that of oestradiol. This finding was considered to be in agreement with other studies that demonstrated low oestrogenic activity of these chemicals *in vivo*. However, they commented that these chemicals are unlikely to have the same effect in natural conditions given their very low activities. Of the phytoestrogens tested, all were active, with potencies approximately 1000- to 2000-fold less active than oestradiol. The inhibitory action of tamoxifen was clearly shown, whilst cortisol did not induce vitellogenin.

Jobling and Sumpter and White *et al.* successfully detected marked differences in activity for the different APEs tested, whilst in the paper by Sumpter and Jobling data demonstrated that the test system would show an enhanced responsiveness to mixtures of chemicals given at submaximal concentrations.

DETAILED REVIEW OF OTHER NON-REGULATORY TEST METHODS

This section presents in detail the history, methodology and endpoints of the remaining published, non-regulatory test methodologies assessed during the course of the review process. These methods are again presented in four major categories:

- *In vivo* systems relevant to assessment of potential toxicity to humans.
- *In vitro* systems relevant to assessment of potential toxicity to humans.
- *In vivo* systems relevant to assessment of effects on wildlife.
- *In vitro* systems relevant to assessment of effects on wildlife.

Systems included in the wildlife categories are restricted to those that were considered unlikely to contribute significantly to the assessment of human health effects. Some of the methods and endpoints included in the “relevant to humans” sections are clearly also of potential relevance for ecological risk assessment.

In line with current concerns, attention has been principally focused on test models that assist in the identification of chemicals with agonistic and/or antagonistic oestrogenic or androgenic activities. A number of the models reviewed were found to have endpoints relating either to more general reproductive health or to other endocrinologically significant aspects, *e.g.* aromatase inhibition. The various types of test are presented in the following three subsections within each of the above major categories:

- Non-specific.
- Oestrogen-related.
- Androgen-related.

The critical assessments of these methods are in Section Critical Assessment of Non-Regulatory Test Methods.

**IN VIVO SYSTEMS RELEVANT TO ASSESSMENT OF POTENTIAL TOXICITY TO HUMANS
NON-SPECIFIC**

RODENT TERATOLOGY SCREEN

Chernoff and Kavlock (1982) published a paper on an abbreviated *in vivo* teratology screen they developed to simplify the initial screening of chemicals for teratological potential, thus reducing the numbers of full regulatorily compliant teratology studies that have to be performed. The basis of this assay was that prenatal insult would be expected to be manifest postnatally as reduced viability and/or impaired growth.

As a validation exercise, a wide range of chemicals previously investigated for definitive teratology were assessed using this simplified screening method. The results from the different methods were then compared. Chemicals examined included sodium salicylate, carbaryl, dinoseb, toxaphene, vitamin A, cacodylic acid, caffeine, endrin, kepone, 5-bromodeoxyuridine, benomyl, ethylenethiourea, 6-aminonicotinamide, decamethrin, ethylenebisisothiocyanate chlordane, mirex, nitrofen, 2,4,5-trichlorophenoxyacetic acid, trypan blue, nickel chloride, aminopterin sodium selenite, thalidomide, 2,4,5-trichlorophenol, lithium carbonate and hexachloropentadiene

METHOD

As an initial preliminary range-setting study, approximately 60-day-old female CD-1 mice were housed five per cage and dosed for five days at one of five dosage levels, using the same routes as used in the definitive teratology. A minimal toxic dose was assessed.

In the main assay, pregnant mice were singly housed and dosed by the appropriate route on days 8 to 12 of pregnancy at the previously defined minimally toxic dose. This was to permit the dams to recover from any toxicity resultant from treatment before parturition, reducing the danger of confounding effects derived from maternal toxicity.

Treated groups were generally made up of 24-30 dams and control groups contained 24-40 animals. Maternal weight changes were recorded during the course of the pregnancy. Animals were permitted to give birth. On days 1 and 3 of lactation the number of live pups was recorded and the pups weighed. Dead pups were necropsied wherever possible, and dams that failed to litter within three days of the expected duration were killed and given a gross examination including the uterine contents.

Data were analysed by ANOVA, followed by Student's t-test on least-square means. One-tailed tests were applied to the live pups and the number of live pups in a litter was used as a covariate, in order to allow for the effects of different sized litters.

ENDPOINTS/DATA INTERPRETATION

- **Number of pups and pup weight:** on days 1 and 3 of lactation.

In the preliminary studies no toxic effects were seen for two compounds, thalidomide and lithium carbonate. Other toxicity data were therefore used to set levels in these cases.

In the main studies, litter size reductions of 12% or greater were necessary to achieve a level of statistical significance, whilst only 7% reduction in pup weight was necessary. This reflected differences in the variability of the data. Eleven compounds were identified that showed a significant

effect on pup weight on day 1. Six maintained the difference at day 3. Only for caffeine was an effect first seen on day 3. A strong correlation was reported between the results of the definitive studies and these screening assays. Of 15 known teratogens, 13 showed effects on litter size or viability and the other two showed reduced body weights in this assay. There were no false negatives, although two chemicals known to induce none-life threatening changes (supernumerary ribs) were not identified by the screen. Of nine compounds not showing effects in the definitive study, three elicited either litter size or weight effects. This might indicate the presence of functional deficits not detectable in the standard (European/USA) study design and so might not be false positives. In the case of some chemicals (*e.g.* vitamin A and mirex), abnormalities were seen in the dead pups. Finally, this test would not detect chemicals that are not teratogenic in this species, and the test sensitivity might be less for compounds eliciting changes not affecting viability.

RODENT PREGNANCY MAINTENANCE TESTS

Phillips *et al.* (1990) reported on a series of experiments to study various aspects of the pharmacology of norethindrone and ethynyl oestradiol. This included the use of a method derived from work by Saunders and Elton (1959).

This method was designed to compare the pregnancy-maintaining capability of test chemicals in pregnant, ovariectomised animals compared to that of progesterone.

METHOD

Adult female Wistar rats were housed overnight with males (1:1 ratio). The presence of vaginal sperm plugs was considered to denote mating, and that day was considered to be day 1 of pregnancy. On day 8, animals were ovariectomised under anaesthesia; some animals were maintained intact as negative controls for comparison. Rats were subsequently treated with either norethindrone, vehicle control or a positive control, progesterone, by daily subcutaneous injection on days 8 to 17 of pregnancy. Eight animals were assigned to each treatment group. Animals were humanely killed after the last administration and their uterine contents examined. Data were expressed as number of normal fetuses per animal.

SUBSEQUENT DEVELOPMENT

Kuhnz and Beier (1994) used a similar model system to investigate the progestational activity of norgestimate and levonorgesterel. In this study pregnant rats were assigned to 10 groups with six animals per group. Animals were ovariectomised on day 8 of pregnancy and dosed two hours later, with the relevant test material at one of a range of dosages or vehicle alone. Animals given either test material also received a daily dose of oestrone. Dosing continued daily for a further 13 days.

Animals were weighed on days 1, 8 and 22 of pregnancy. One day after the last treatment, animals were humanely killed and the number of live fetuses assessed. The degree of pregnancy was expressed as a percentage of the fetuses in the control animals.

ENDPOINTS/DATA INTERPRETATION

- **Number of normal fetuses:** data collected for each animal (expressed as a percentage of controls by Kuhnz and Beier).

Phillips *et al.* (1990) reported that treatment with norethindrone at the lower dosages used permitted maintenance of pregnancy, whilst numbers were reduced at higher levels. In the study by Kuhnz and Beier (1994), a difference in progestational activity of three- to 10-fold was found between the two chemicals tested, with dosage relationship being clearest for levonorgestrel. In the case of norethindrone, there was evidence of fetal loss at the highest dosage examined.

RAT IMPLANTATION ASSAY

Dhar and Mattu (1995) reported on a model used in the assessment of chemicals for potential female contraceptive potency. In this study, two hydroxynaphthaquinones (2-hydroxy-3-methyl-1, 4-naphthoquinone monosemicarbazone and 2-hydroxy-1, 4-naphthoquinone monothiosemicarbazone) were screened for anti-implantational activity in the rat.

METHOD

Albino rats of the Charles Foster strain were used. Females were housed with males of proven fertility on the evening of the proestrus stage. Mating was assessed by vaginal smearing the following morning; the presence of sperm was taken to denote day 1 of pregnancy. Females were given the appropriate dosage of test material or vehicle (five animals per treatment group; 15 in the control group) by oral gavage from day 1 to 11 of pregnancy. On day 11 animals were laparotomised and implantation sites recorded. Animals were permitted to go to term and the pups were maintained for one month to permit observation for developmental abnormalities.

ENDPOINTS/DATA INTERPRETATION

- **Number of implantation sites:** by visual observation during laparotomy on day 11.
- **Pup abnormality:** by visual observation.

Dosage-related reduction in numbers of implantation sites were reported for both chemicals at laparotomy on day 11 of pregnancy. Subsequently, after delivery no gross teratogenic effects were observed.

RODENT VAGINAL ASSAY

The rodent vaginal assay is generally regarded as a highly specific assay for oestrogenic activity. The variations of the basic test are reviewed in detail in Section 0 below. Deckers and Schuurs (1989), however, proposed a modification to this model to permit its use in identifying chemicals with aromatase-inhibiting activity. The various experiments conducted during the development of this system are reviewed here.

The model was based upon either surgically or chemically hypophysectomised (hypx) rats treated with an oestrogen precursor, immediately followed by administration of the test chemical. The endpoint was vaginal cornification, monitored by daily vaginal smearing.

METHOD

Mature female Hsd/Cbp: ORGA rats (200-250 g) were obtained from a commercial source. After an initial acclimatisation period, animals were hypophysectomised using the parapharyngeal route and given post-operative glucose. During the week after surgery, daily vaginal smears were prepared, stained with Giemsa solution and evaluated. Animals with positive smears (*i.e.* those with greater than 50% nucleated or cornified epithelial cells) were excluded from further study.

In the initial experiment, after the one-week recovery/assessment period animals were dosed with the oestrogen precursor dehydroepiandrosterone-3-sodium sulfate (DHEAS) by daily oral gavage for four days, and vaginal smears were prepared on the sixth day after commencement of dosing. Results were expressed as percentage positive versus total number of smears.

In a second experiment, animals were given a similar treatment regimen four, six and eight weeks after hypophysectomy. To assess the ability of the system to detect aromatase-inhibiting activity, a series of test chemicals were given by subcutaneous injection immediately following DHEAS or alternative oestrogen precursor treatment. These included 9-mercapto-androstenedione, 4-hydroxyandrostene-dione and 19-methylene-androstenedione.

In addition, the effect of using chemical hypophysectomy was compared with the surgical model by administration of an LHRH-antagonist (ORG 30276) to mature female rats from the first day of oestrus for four days. On the fourth day, DHEAS and an aromatase inhibitor were also administered.

The paper also presented studies on the oestrogenic and anti-oestrogenic activity of various chemicals using a vaginal smear method (see relevant section below).

ENDPOINTS/DATA INTERPRETATION

- **Induction of oestrus:** assessed by examination of vaginal smears

DHEAS treatment one and four weeks after surgical hypophysectomy induced signs of cornification by the third day of treatment which continued for up to four days. Beyond this length of time after hypophysectomy, responsiveness diminished. Dosage relationship was apparent up to a maximal response at 16 mg/kg. Daily administration of the various test chemicals showed that they possessed a wide range of activities. The investigations of chemical induction of hypophysectomy showed that it was possible to obtain similar vaginal responses to the surgically induced model. The chemical

induction method offered a means of reducing stress to the animals and was also technically less demanding.

RODENT BEHAVIOURAL MODIFICATION

In this section, two models used to investigate hormonal control mechanisms on mating behaviour in the adult hamster and rat are reviewed. A mouse model assessing behavioural changes in adults arising from modulation of oestrogenic stimulation during their fetal period is presented in the “oestrogen-related” section below.

Hsu (1990) investigated the influence on lordosis of the treatment of female hamsters primed with oestrogen benzoate (EB), testosterone propionate (TP), with an aromatase inhibitor, androst-1, 4, 6-treiene-3, 17-dione (ATD), or with the oestrogen antagonist tamoxifen (TAM). This work was based on lordosis having been shown to be induced by treating normal female hamsters with testosterone and progesterone. It attempted to assist in the clarification of the hormonal interactions responsible for the control of reproductive behaviour patterns in the rodent.

Menard *et al.* (1992) studied (in a series of experiments) the modulating effects of oestrogen on the inhibitory effect of scopolamine (a cholinergic muscarinic receptor blocker) on the lordosis response in female rats.

METHOD

Hsu (1990): Ovariectomised female hamsters were assigned to six groups of eight animals each. At approximately 120 days of age, all animals commenced a four-day treatment regimen. Animals were given TP on the first day and progesterone (P) on the last. Treatments on days 2 and 3 varied between groups as follows:

TP + ATD
 TP + TAM
 TP + Vehicle
 EB + ATD
 EB + TAM
 EB + Vehicle

Animals were dosed by subcutaneous injection between 12:00 and 14:00 hours daily. Behaviour test were conducted under fluorescent light in a soundproof room between 18:00 to 22:00 hours on the day of P injection. Females were paired with a sexually proven male hamster for five minutes in a glass aquarium containing wood chip bedding. The occurrence and duration of lordosis was recorded by an event recorder. Data was subject to one-way ANOVA and Scheffé’s test.

Menard *et al.* (1992): Long-Evans hooded female rats were housed individually under standard laboratory conditions.

In the initial experiment 40 females were ovariectomised and, following a two-week recovery period, were injected subcutaneously with varying dosages of oestradiol benzoate. Animals were subject to behavioural testing (see below) after 24, 48 or 72 hours. Four hours before each assessment, animals received a subcutaneous injection of progesterone. Immediately following the initial behavioural test, females were dosed intraperitoneally with scopolamine hydrochloride or vehicle alone and re-tested 15 minutes later. Three days after this initial test, females were subject to the same treatment regimen and

testing except that all animals received the scopolamine (*i.e.* none received vehicle alone on this occasion). This procedure was adopted to investigate whether any reduction in scopolamine effectiveness was attributable to repeated exposure to the oestrogen or scopolamine. It was repeated in week 3 to further investigate any time course changes. In the fourth week, in order to investigate the potential influence of previous high-level exposure to oestrogen, all animals received only the lowest dosage of oestrogen in combination with progesterone and all were given scopolamine treatment.

In the second experiment, animals were subject to a similar initial protocol. Ovariectomised animals received various doses of oestrogen and were examined at the same times after dosing. Progesterone priming was again used, and animals were assessed before and after receipt of either scopolamine or vehicle. This time, however, different dosages of scopolamine were investigated. Three days after the first behavioural test, the week 1 protocol was repeated except that those initially given saline rather than scopolamine were this time given the high dosage of scopolamine. This treatment was repeated in weeks 3 and 4, with only the low dosage of oestrogen being administered in week 4.

Test arenas comprised glass aquaria (50 × 25 × 30 cm) with Plexiglas lid and wood shaving bedding. Females were introduced singly into arenas containing a male rat, and the incidence of lordosis during a 10-minute pairing period was recorded. The lordosis quotient was calculated for each session, as:

$$(\text{number of lordoses/number of mounts}) \times 100$$

These were converted into percentage of control values, which were analysed using a 2 × 3 × 4 (drug × oestrogen × week) analysis of variance with repeated measures followed by Newman-Keuls pairwise comparisons.

ENDPOINTS/DATA INTERPRETATION

Hsu (1990):

- **Total lordosis time:** calculated for each group.

The total lordosis times for groups receiving EB + vehicle, EB + ATD or TP + vehicle, were all longer than 200 seconds, and were not significantly different from each other, but they were significantly different from the other groups. The time for the group given EB + TAM was 117 seconds, whilst the TP + TAM and TP + ATD groups displayed almost no lordosis. These results showed that the ability of TP + P to activate lordosis in female hamsters was mediated through aromatisation of testosterone into oestradiol. Thus inhibition by ATD abolished the activity. The absence of inhibition in oestrogen-primed animals confirmed that the response seen was not attributable to side-effects of ATD.

Menard *et al.* (1992):

- **Lordosis quotient:** calculated for each animal as detailed above and expressed as percentage of control values.

Data for week 1 of the first experiment confirmed the inhibition of lordosis by scopolamine, when compared with the controls. However, the effect was less marked in those primed with the highest level of oestrogen. The effectiveness of scopolamine was reduced in the second and third week, irrespective of exposure to saline or scopolamine in week 1, *i.e.* the effect probably results from repeated oestrogen exposure rather than from repeated scopolamine administration. In the last week,

the data indicated that the effects of exposure to previous high dosages of oestrogen were still apparent in animals despite all of them being given low dosages on that occasion.

In the second series of experiments, it was shown that the reduction in scopolamine's effectiveness in the second week of the study was independent of prior exposure to this chemical and that the effect was maintained in subsequent weeks.

Inhibition of lordosis was clearly associated with muscarinic receptor blockade but was influenced by prior oestrogen exposure level. This effect was reported to be persistent over several weeks. The influence of progesterone was not investigated in these experiments. Several factors may influence responsiveness, including muscarinic receptor levels, levels of endogenous transmitter, cholinergic enzyme activity changes, modification of other neurotransmitter systems, or even changes in metabolic capacity.

OESTROGEN-RELATED

OVARIECTOMISED PRIMATE MODEL

On the basis of their earlier work and that of other authors, Schane *et al.* (1972) developed an *in vivo* model for the evaluation of oestrogenic chemicals using non-human primates. The test was based on treatment of ovariectomised monkeys with an oestrogenic chemical, which resulted in menstrual-like bleeding following withdrawal of treatment. The authors reported a series of experiments with this model conducted over a four-year period.

METHOD

Female rhesus monkeys, *Macaca mulatta*, weighing 2-4 kg, were bilaterally ovariectomised and subsequently maintained under standard laboratory conditions. Animals were assigned to one of four groups, with four animals per group. For each study cycle, monkeys were dosed daily with one of a range of dosages of a test chemical for 10 days. Animals were vaginally smeared daily to detect the onset of menstrual bleeding. In addition, on the first day of treatment and the day after the last dose, the colour of the perianal sexual skin was subjectively rated on a 0-4 scale. Between testing of each chemical, monkeys received 17 β -oestradiol daily for 10 days, after which withdrawal bleeding was monitored. The monkeys consistently showed bleeding approximately nine days after withdrawal of 17 β -oestradiol treatment. Bleeding was never seen sooner than day 6 after treatment and, in all but two instances, occurred by day 15. On this basis, it was considered that prior treatment history did not bias the response to any given treatment and, that animals could therefore be reused for screening different chemicals.

17 β -oestradiol was administered subcutaneously, whilst the other chemicals investigated (oestrone, ethynyl oestradiol, mestranol, Premarin, diethylstilboestrol and prepyrol) were administered by oral gavage. The day of withdrawal, bleeding following a constant dose of 17 β -oestradiol was analysed with respect to individual bodyweight using the Spearman Rank Correlation test. No significant correlation was found, and therefore all doses were administered on a per animal basis.

SUBSEQUENT DEVELOPMENT

Paliwal *et al.* (1992): The ovariectomised rhesus monkey was also the animal model in a method used by Paliwal *et al.* (1992) to investigate the effects of the anti-oestrogens centchroman and tamoxifen on fallopian tube protein patterns. In this study, ovariectomised animals received one of the following treatment regimens for seven days:

Group	Treatment
1	vehicle control
2	17 β -oestradiol
3	Centchroman
4	Tamoxifen
5	17 β -oestradiol + centchroman
6	17 β -oestradiol + tamoxifen

Animals were humanely killed 24 hours after the last dose and the fallopian tubes dissected. Each tube was flushed with saline, and the pooled flushings were centrifuged and deep-frozen. Flushings were analysed for total protein content using the method of Bradford, with Bovine Serum Albumin as standard, and were also sodium dodecyl sulfate-polyacrylamide gel electrophoresed, followed by periodic acid and Coomassie brilliant blue staining. Staining patterns were scanned at 560 and 550 nm.

ENDPOINTS/DATA INTERPRETATION

Schane *et al.* (1972):

- **Withdrawal bleeding:** by vaginal smear examination.
- **Sexual skin colour:** by observation.

For each chemical, the dose resulting in withdrawal bleeding for all animals in a group and the dose that was completely ineffective at inducing bleeding were identified. The doses initiating or not affecting sexual skin colour were also recorded.

The relative potencies of the test chemicals in this study generally correlated with those previously found for humans, although both oestrone and Premarin were more potent in the rhesus monkey than in women. It was suggested that this might reflect interspecies differences in the rates of conversion of the chemicals to oestradiol. The dosages initiating perianal sexual skin colour change did not correlate with those eliciting withdrawal bleeding. It was considered that this would provide an alternative endpoint to enable uterotrophic activity to be evaluated separately from other oestrogenic effects.

Paliwal *et al.* (1992):

- **Total protein content:** using the method of Bradford.
- **Electrophoresis staining patterns:** by scanner.

The various treatment regimens elicited a differential response for protein macromolecules, with changes being detected in the 130K, 95K, 90K and 85K proteins. Evidence of both agonistic and antagonistic properties was recorded for centchroman and tamoxifen.

RODENT UTERINE ASSAYS

Over the last 60 years, oestrogenic or anti-oestrogenic activities of chemicals have frequently been assessed by models that were based on uterotrophic responses in the rodent. Early methods used uterine weight as an effective, reliable and easy-to-record endpoint. Subsequently, methodology has been refined and a number of alternative endpoints have been developed. The rat models are reviewed Section x on Rat Uterine Models. Mouse models and a rabbit-based model (although it is not a rodent) are discussed below.

MOUSE MODELS

In addition to the various rat-based assays discussed above, many workers have used the mouse as the basis for similar uterotrophic assay methods.

In a series of experiments, Folman and Pope (1966) studied the interactions between various oestrogens using the uterotrophic and vaginotrophic responses in mice. The vaginotrophic aspects of their work are reported in the relevant section below.

The effects of co-administration of a potent oestrogen (such as 17 β -oestradiol or diethylstilboestrol) with a weak oestrogen (such as coumesterol, genistein and dimethylstilboestrol) or progestin (such as norethisterone acetate or megestrol acetate) were studied as were the combinations of oestrone with oestriol, or oestriol with genistein.

METHOD

Randomly bred female albino BSVS mice (three to four weeks of age) were allocated to groups using a randomised block design with nine mice per group.

The test materials were administered by subcutaneous injections over a three-day period. The combinations of chemicals were given at separate injection sites, one injection immediately after the other. The daily treatment regimens varied depending on the chemical; some chemicals were given as equal sized, twice-daily injections, whilst others were given once a day with the second daily injection being vehicle alone. The precise treatment regimen depended upon solubility and toxicity considerations.

Seventy to 74 hours after the first administration, animals were weighed and the uteri placed in physiological saline. After careful drying on blotting paper, uteri were weighed. In one experiment the uteri were left for one hour in an atmosphere saturated with water vapour before weighing. All uterine weights included the fallopian tubes.

SUBSEQUENT DEVELOPMENT

Uterine weight

Dorfman and Kincl (1966): reported on the relative uterotrophic activity of a series of steroids (including 3- and/or 17-deoxy derivatives of oestrone and 17 β -oestradiol and selected alkyl derivatives) when administered to mice by subcutaneous injection and/or oral gavage to mice for three days.

Swiss albino female mice (21 to 23 days of age) were treated once daily by the appropriate route for three days. Twenty-four hours after the last administration, animals were humanely killed and the uterine weight and bodyweight recorded. Oestrone was used as a positive control in each of a series of experiments with the various test chemicals, and results were expressed as a percentage relative to its potency (considered to be 100%). Values were determined graphically from a standard oestrone log-dose response curve. Statistical calculations were by the Emmens method.

Thigpen *et al.* (1987a, b, c) documented a standardised method for the conduct of a mouse bioassay in a series of papers. Studies were conducted with CD-1 mice to determine the appropriate weaning age and length of bioassay. Litters were standardised at one to two days of age to eight female pups. Uterine growth curves were generated from mice weaned at day 15 of age and fed unmedicated diet for varying periods up to day 28 (time points investigated were days 15, 20, 22, 24, 26 and 28 of age). Pups were housed in groups of eight per cage under standard conditions during this period. On completion of the appropriate period, animals were killed and the uteri removed onto blotting paper. Six to eight pinpoint holes were made in the horns with the tip of a scalpel blade, and the intrauterine fluid gently expressed. The uterine weight was then recorded to the nearest 0.1 mg within 10 seconds of trimming and blotting. In addition, uterine weights were recorded for females of 15 to 40 days of age at 30-second intervals over a three-minute period to determine the potential effect of atmospheric drying. The effects of various commercial diets were also studied, using animals weaned at 15 days of age, with the uterine bodyweight ratios being determined after three, five and seven days of feeding.

In a further series of experiments, pups were weaned at 15 or 17 days of age, housed in cages of five animals, and assigned to one of three treatment regimens. Animals were fed diet containing either 0, 4 or 6 ppb diethylstilboestrol until they were 24 or 26 days of age. Bodyweight and uterine weight ratios were calculated at five, seven, nine or 11 days post-weaning.

The experiments were conducted using a single blind design, with assignment of animals to groups by a computerised random number generator. A group size of 15 animals was generally used. An outlier test was conducted to identify values more than two empirical interquartiles away from the mean; these were excluded from further calculations. Data were subject to analysis of variance and Student's t-test.

Plass *et al.* (1990) used an ovariectomised mouse uterotrophic model to study the potential oestrogenicity of various fava bean extracts when administered via the diet for four days.

Juvenile mice were ovariectomised and randomly assigned to groups of 10 or 11 animals (housed five or six to a cage) and were fed diet containing the various extracts or diethylstilboestrol (positive control), as follows:

Group	Diet
1	standard - untreated
2	5 µg diethylstilboestrol/kg diet
3	10 µg diethylstilboestrol/kg diet
4	25 µg diethylstilboestrol/kg diet
5	fava bean flour
6	protein isolate (acetylation degree 97%)
7	protein isolate (acetylation degree 88%)
8	protein isolate (acetylation degree 74%)
9	protein isolate (acetylation degree 29%)
10	unmodified protein isolate

On the fifth day of treatment animals were weighed, killed, exsanguinated, and the uterine weights recorded. Uterine weights were expressed as absolute and bodyweight-relative values.

Galey *et al.* (1993) performed a series of experiments to establish the use of the mouse uterine bioassay in assessing the oestrogenic activity of forages.

Prepubertal female Swiss-Webster mice (10-12 g) were assigned to groups (three animals per group) and fed test diets at the rate of 20 g/group/day for five days. A control group and a known spiked (positive control) group were included in each experiment.

Animals were killed on day 6 of study and bodyweight and uterine weights recorded. Samples were taken into buffered formalin in some instances for subsequent histopathological examination. Throughout the study bodyweight-relative values were found to parallel the absolute data and therefore only the absolute values were considered further.

Data were analysed by Bartlett's test for homogeneity of variance. This was followed by one-way analysis of variance, with initial log transformation if Bartlett's test was significant.

The sensitivity of the test system was initially established by administration to mice of diethylstilboestrol or oestradiol, at a range of concentrations, either in standard mouse diet or by adding the acetone extract from spiked hay to diet and allowing it to dry before feeding to the mice. Other investigations included the similar addition of coumesterol directly to diet or as a spike to hay, followed by extraction. A sample of clover forage known to have caused clinical hyperoestrogenism was also investigated.

In addition to these models, Bigsby and Young (1994) used uterine weight recording as well as a number of biochemical markers of oestrogenicity. This paper is considered in the following section.

Uterine biochemistry/receptor binding

Bigsby and Young (1994): As part of a series of investigations on the oestrogenic effects of the antiprogesterin onapristone, uterine epithelial DNA synthesis in neonatal mice was studied, and uterine weight and progesterone and oestrogen receptor levels in the ovariectomised adult.

Neonatal (four-day-old) BALB/c mice were given intraperitoneal injections of dexamethasone or progesterone alone or in combination with varying dosages of onapristone or other antiprogesterins. Dosages were adjusted for bodyweight. Fifteen hours later animals were injected with tritiated thymidine. Animals were killed one hour later and uterine specimens formalin-fixed and paraffin-embedded. The

thymidine-labelling index was derived from autoradiography sections and expressed as epithelial labelling index.

Juvenile adult ICR mice were ovariectomised at four or eight weeks of age. After a two-week recovery period, animals received the test chemical by daily subcutaneous injection, with adjustment of dosage for bodyweight, for three days. Twenty-four hours after the last injection animals were killed, their uteri weighed, and a cytosolic extract prepared by homogenisation and centrifugation. Aliquots were added to tubes containing increasing concentrations of tritiated oestradiol with or without a 500-fold excess of unlabelled oestradiol. After 18 hours of incubation at 4°C, free radiolabelled steroid was stripped using dextran-coated charcoal and radioactivity in the aliquot was determined to enable derivation of oestrogen receptor levels. Progesterone receptor levels were similarly assessed using tritiated 16 α -ethyl-21-hydroxy-19-norpregn-4-en-3, 20-dione in the presence or absence of excess progesterone. Receptor concentrations were determined by extrapolation from Scatchard plots of binding data.

Statistical analysis comprised t-test or analysis of variance, followed by testing of individual means against the control value with Fischer's least-squares difference method or testing among means with Scheffé's F test.

Crombie *et al.* (1994) published a paper on the use of a mouse uterus model with creatine kinase activity as a marker for unopposed oestrogen activity. On the basis of published literature, the brain isoform of creatine kinase (CK) is one of the major proteins induced in the uterus of the rat, but not that of humans, in response to oestrogenic stimulation. In this study a mouse decidualisation model was used and CK activity looked at as a cellular marker to assess the oestrogen action during progesterone withdrawal or receptor blockade.

Mature virgin female BALB/c mice (10-12 weeks of age) were maintained under normal environmental conditions. At the start of the experiment, females were co-housed overnight with vasectomised males of the same strain (pseudopregnancy was dated from the morning when a vaginal plug was detected). On day 3 of pseudopregnancy, animals were treated with vehicle alone: progesterone or one of two test chemicals (antiprogestins). At 16:00 hours the next day, a decidual stimulus, sesame oil, was injected intraluminally into the right uterine horn of each animal. Animals were necropsied 72 hours later and the uteri removed, blotted and weighed before deep-freezing pending assay. Weight gain was calculated by subtracting the weight of the non-stimulated horn from that of the stimulated horn.

Uterine samples were thawed on ice, homogenised and centrifuged at 45,000 rpm for 75 minutes at 4°C. The supernatant was collected, diluted 1:10, and analysed for CK activity using a commercial kit with measurement by spectrophotometry. Values were expressed relative to protein concentrations obtained by the method of Bradford.

Data were analysed by Student's t-test.

Pentecost and Teng (1987) worked to identify an oestrogen-inducible secretory protein that had previously been detected and isolated from the mouse uterus. In this paper, they reported on the isolation and cloning of a phage of the cDNA for the protein by screening a mouse uterine cDNA library using an antibody to the oestrogen-induced uterine protein. RNA from uteri of control mice and mice treated with diethylstilboestrol for three days was probed using the appropriate DNA clone to confirm inducibility of the corresponding mRNA. A weak but detectable signal was found in the control material, whilst a 300-fold induction was found for the treated samples. Sequencing of the cDNA was undertaken and the results

compared to various known compositions for transferrins. From this work the homology of the induced protein to lactoferrin was established.

In later work, **Nelson *et al.* (1991)** studied the interactivity of epidermal growth factor (EGF) and oestrogens in the *in vivo* stimulation of genital tract growth and differentiation in the female mouse. Included in this paper was the use of immunohistochemical, immunoblot and polymerase chain reaction methods for the analysis of uterine lactoferrin induction. In this experiment, eight-week-old-mice that had been ovariectomised for at least three weeks were given pellets of EGF, diethylstilboestrol or control material implanted under the kidney capsule. After 48 hours' exposure, the mice were killed. For the immunohistochemistry investigation, uteri were fixed in Bouin's solution, paraffin embedded, incubated with rabbit antibody specific for mouse lactoferrin and then with an alkaline-phosphatase-conjugated secondary antibody. The uteri and vaginas from other animals were homogenised, centrifuged at 100,000 g for 45 minutes, and stored deep-frozen until use for immunoblotting analysis. Aliquots of the tissue samples, together with purified mouse lactoferrin, were fractionated by SDS/8% PAGE and transferred to nitrocellulose. Detection was by incubation with affinity-purified antibody against mouse lactoferrin and an [¹²⁵I]-labelled donkey anti-rabbit antibody. Polymerase chain reaction analysis involved the isolation of total RNA from the uteri of each group. A positive control was used by deriving RNA from lactating mouse mammary glands. Total RNA was reverse-transcribed with a commercial reverse transcriptase and the cDNA amplified using a commercial kit. After repeated washing and centrifuging, samples were electrophoresed and stained with ethidium bromide.

ENDPOINTS/DATA INTERPRETATION

Folman and Pope (1966); Dorfman and Kincl (1966); Thigpen *et al.* (1987a, b, c); Plass *et al.* (1990); Galey *et al.* (1993):

- **Uterine weight:** expressed as:
 - absolute values (Folman and Pope, 1966; Galey *et al.*, 1993);
 - potency relative to oestrone (Dorfman and Kincl, 1966);
 - bodyweight-relative values (Thigpen *et al.*, 1987 a, b, c);
 - absolute and bodyweight-relative values (Plass *et al.*, 1990).

Folman and Pope demonstrated that the weak oestrogens coumesterol, genistein and dimethylstilboestrol, and the progestins norethisterone acetate and megestrol acetate, were all weakly uterotrophic with only genistein giving a steep dose-response curve similar to that of the most potent oestrogens. The authors commented that the percentage increase in weight responses seen differed for the uterus and vagina. However, responses varied between chemicals such that it was not possible to define one of the endpoints as consistently more sensitive than the other. Evidence of inhibition of the effects of strong oestrogens on the uterus was apparent with co-administration of some of the weaker oestrogenic chemicals. Dorfman and Kincl expressed their results in terms of potency relative to a standard (oestradiol) and showed that only two of the steroids investigated were equal to, or more active than, this standard. These were 17 β -oestradiol benzoate and 3-cyano-17 α -ethinyloestra-1,3,5(10)-trien-17 β -ol. Differences in activity were also seen to depend upon the route of administration.

Data from the studies by Thigpen *et al.* indicated that some normal mice experience a rapid increase in uterine growth as early as day 23 or 24 of age. The authors therefore recommended that bioassays be

conducted on animals younger than this. They suggested that weaning at day 15, followed by a seven-day treatment period, was preferable because at this age the uterine weights are more uniform and the mice have not been exposed to much solid food potentially containing exogenous oestrogens. Indeed, different commercial diets were shown to result in differences in the uterus-bodyweight ratios of weanling mice. These differences were largely attributed to the fat and carbohydrate composition of the diets. Such effects underline the importance of control of dietary composition if comparisons across different assay occasions are intended. In addition, the need to establish narrow, carefully controlled age ranges for weanlings was stressed. Overall, the authors suggested that a standardised method should incorporate the following:

- a) Strain and stock must be specified.
- b) All mice must be of the same age.
- c) Mice should be free of infectious disease.
- d) Bioassay period should end before normal oestrus begins.
- e) Standardised control diets should be used.
- f) Diethylstilboestrol at 6 ppb should be used as a control.
- g) Animals should be randomly assigned to groups.
- h) Initial body weights should be recorded and animals failing to gain weight excluded, assuming that administration is via the diet (*i.e.* these animals are considered unlikely to have eaten the medicated diet if they are not gaining weight).
- i) Standardised necropsy and weighing procedure are needed.
- j) In order for a bioassay to be valid, a statistically significant difference in uterus-body weight ratio must be established between the negative and positive control groups.

Plass *et al.* demonstrated a dosage-dependent increase in absolute and bodyweight-relative uterine weight for ovariectomised animals given diethylstilboestrol, with effects clearly apparent in the 5 µg diethylstilboestrol/kg diet group.

Effects on absolute uterine weight were apparent for animals given the various fava bean products, although this was attributed to reduced food intake and growth with a change in bodyweight-relative values only being seen in Group 9. It was therefore considered that these extracts were without oestrogenic activity. Galey *et al.* reported that diethylstilboestrol, oestradiol and coumesterol all caused significant increases in uterine weight in juvenile non-ovariectomised mice. The magnitude of enlargement became more variable as the dosage increased. This, together with the results of the Bartlett's analysis, was suggested as indicating data inhomogeneity, although transformation of data overcame the problem. Limited histopathological examination showed the increase in uterine weight to be associated with hyperplasia, cellular infiltration and oedema. The test system was shown to be capable of identifying feeds that had reportedly affected fertility in livestock, and therefore it was considered that this method would be of value as an alternative to chemical analysis of any forages considered to have potentially oestrogenic activity.

Bigby and Young (1994):

- **Uterine epithelial DNA synthesis:** measured in neonatal mice by autoradiography and expressed as epithelial labelling index.
- **Uterine weight:** expressed as absolute values for ovariectomised adults.

- **Uterine progesterone and oestrogen receptors levels:** determined, in the ovariectomised adult, using radiolabelled materials and extrapolation from Scatchard plots of binding data. Relative binding affinity calculated as the concentration required to displace 50% of the bound tritiated material, relative to the concentration of unlabelled substrate that produced such a displacement.

In neonatal animals, progesterone and dexamethasone maximally inhibited DNA synthesis at 40 and 1 µg/g, respectively, although the effects were found to be overcome by onapristone co-administration at 0.1-40 µg/g. The onapristone was also found to stimulate DNA synthesis when administered alone. Six-week-old ovariectomised mice showed increases in cytosolic progesterone receptor levels in response to three days of onapristone treatment, despite the absence of any effect on absolute uterine weight. In contrast, when 10-week-old animals were studied, effects on both these endpoints were seen. Oestradiol treatment was similarly observed to affect uterine weight and oestrogen receptor levels, whilst onapristone also affected the expression of these receptors.

Crombie *et al.* (1994):

- **Uterine weight gain:** calculated by subtracting the weight of the non-stimulated uterine horn from that of the stimulated horn.
- **Uterine CK activity:** assessed by spectrophotometry; expressed relative to protein content.

A marked increase in uterine horn weight in response to decidualisation was reported, which was blocked by treatment with the antiprogestones. The decidualised horn was also found to have a lower CK activity than the control (undecidualised) horn in the control animals. This was taken as evidence for the suppression of oestrogenic activity by the process of decidualisation. The difference in CK activity between horns was found to be abolished by the anti-progestational agents. It was suggested that this model would therefore be of value for the determination of oestrogenic activity and the detection of unopposed oestrogen action associated with progesterone antagonism.

Nelson *et al.* (1991)

- **Lactoferrin levels:** using immunocytochemical (uterus only), immunoblotting (uterus and vagina) or polymerase chain reaction (uterus only) methodologies.

EGF and diethylstilboestrol elicited similar induction of lactoferrin and the associated lactoferrin mRNA in the mouse uterus and therefore concluded that responses normally associated with oestrogenic stimulation may arise from stimulation with certain peptide growth factors in the absence of oestrogens.

RABBIT

As part of a series of experiments on the pharmacology of norethindrone and ethynyl oestradiol, Phillips *et al.* (1990) studied the hormonal responsiveness of the rabbit endometrium. Although the test protocol detailed below was intended to identify progestational rather than oestrogenic activity, it is clear from the result that the rabbit endometrium is sensitive to oestrogenic stimulation, suggesting that this species might be suitable for use in situations where either the rat or mouse prove to be unsuitable models.

METHOD

Immature New Zealand white female rabbits (900-1200 g) were injected subcutaneously with 17β -oestradiol daily for six days. From the next day, the rabbits received the test material by daily oral gavage for five days. One day after the last dose, animals were killed and uteri excised, fixed and processed histologically. The degree of endometrial stimulation was assessed microscopically.

ENDPOINTS/DATA INTERPRETATION

- **Endometrial stimulation:** assessed microscopically.

The test material stimulated the endometrium in the immature oestrogen-primed rabbit in a dose-related fashion.

RODENT ENDOMETRIOSIS ASSAY

Vernon and Wilson (1985) investigated a series of methods for the surgical induction of endometriosis in the rat. This work was intended to establish a non-human model for the study of the pathophysiological attributes of this condition. However, the suitability of such a model system for investigation of endocrinologically active chemicals was recognised by other workers. The surgical method established by Vernon and Wilson is described in detail below.

METHOD

Mature (70-day-old) Sprague-Dawley female rats were allowed to acclimatise to standard housing conditions for a one-month period, during which vaginal cytological cyclicity was monitored. Only animals exhibiting four or more consecutive oestrus cycles of either four or five days were used on study.

On the morning of the study, proestrus animals were anaesthetised. Under aseptic conditions, a 2-3 cm mid-line incision was made into the ventral abdominal cavity and the reproductive tract examined to confirm oestrus cycle stage. The distal 2 cm of the right uterine horn with associated ovary was ablated, trimmed of excess fat and longitudinally bisected. Square sections of 1-2 mm were obtained. Three of these were attached to the utero-ovarian ligament and one to the utero-ovarian ligament [ed?]. Implants were sutured to the peritoneal cavity, with the uterine serosal layer in direct apposition to the peritoneal surface. Mesenteric implants were attached adjacent to mesenteric blood vessels; utero-ovarian implants were affixed to the fat pad of the utero-ovarian ligament. Subsequently, the abdominal cavity was irrigated with 1.0 mL of sterile saline and the abdomen closed.

In order to establish the suitability of this method, serial re-examinations were performed on the first day of oestrus after surgery and then at 14, 20, 30 and 40 days.

The effects of pregnancy following endometrial implantation were also investigated.

SUBSEQUENT DEVELOPMENT

Alternative rat model

Henig *et al.* (1988):

Although Jones (1984) established that such a model allowed investigation of the effects of drugs on endometriosis, Henig *et al.* (1988) developed a modified system to overcome potential problems with chemicals causing ovarian suppression or directly acting on the endometrial tissue. In this modification, female rats were subject to endometrial implantation using a similar schedule. However, 21 days after transplantation the animals were surgically examined for the presence of viable endometrial explants and both ovaries were removed. The animals were then assigned to a series of study groups and received treatment for 21 days by daily subcutaneous injection, as follows:

Group	Treatment and dosage	Expected action
1	intact control/sesame oil	control
2	ovariectomised control/sesame oil	surgical control
3	ethylamide buserelin/20 µg/kg	medical hypophysectomy
4	Danazol/12 mg/kg	pseudomenopause
5	ethynyl E, & norgestrel/1 µg/kg & 10 µk/kg, respectively	pseudopregnancy

After the 21-day treatment period, the animals were again laparotomised. The explants were evaluated for size, macroscopic appearance and fluid content, then excised, and samples retained either in 10% formaldehyde for histological examination or deep-frozen (-70%) pending assay for oestrogen receptor and progesterone receptor levels. Blood samples were also taken at this time for serum oestrogen (E₂) and progesterone (P₄) concentrations. Data were analysed by one-way analysis of variance (ANOVA). A posteriori t-tests were used to determine which treatment groups from the ANOVA contributed to the significant differences among the means.

Mouse model

Cummings and Metcalf (1995) investigated the use of the mouse as a model for endometriosis. On the basis that effects of xenobiotics on the immune system may be important in the aetiology and pathology of endometriosis, the authors believed that a mouse model would be preferable given the better characterisation of that of the mouse immune system compared with that of the rat. The method developed was validated by evaluation of the size and histological appearance of the endometriotic sites, inhibition of explant growth following ovariectomy, and stimulation of growth following replacement with oestrogen after ovariectomy.

Adult female B6C3F1 mice were maintained under standard housing conditions. Surgical procedures closely followed those described by Vernon and Wilson for rats, with the exception that only three pieces of uterus were implanted onto the mesentery of the mouse. Three weeks post surgery, mice were assigned to three groups, each with eight animals, and subjected to the following regimens:

Group	Surgical procedure	Treatment
1	killed, endometrial site diameters measured	not applicable
2	bilateral ovariectomy	0.05 mL corn oil by subcutaneous injection
3	bilateral ovariectomy	0.5 µg oestrone in 0.05 mL corn oil by subcutaneous injection

Where appropriate, treatment commenced on the day of ovariectomy and continued for 21 days before the animals were killed and the endometrial explants examined. Severe adhesions frequently occurred over the sites, necessitating careful dissection. Explants were then measured and fixed in 10% formalin. Following routine paraffin embedding and staining with haematoxylin and eosin, samples were examined histopathologically. Data for explant size were analysed by ANOVA, with post hoc comparison between groups using t-test.

ENDPOINTS/DATA INTERPRETATION

Rat models:

Vernon and Wilson (1985):

- **Explant length** (mm): method of measurement not documented.

Explants consistently developed into highly vascular, tear-shaped cysts without any histological necrosis and grew to approximately 5.0 mm by 60 days after surgery. This size was subsequently maintained, and explant length was found to be a satisfactory endpoint. It was shown that pregnancy suppressed but did not ablate the explants, whilst the presence of explants had a negative impact on fertility.

Henig *et al.* (1988):

The following endpoints were used:

- **Explant length** (mm): method of measurement not documented.
- **Histological appearance of explant endometrium:** appearance of the endometrial glands and stroma assessed.
- **Serum E₂ and P₄ hormone levels:** assessed using proprietary radioimmunoassay kits.
- **Endometrial ER and PR receptor levels:** assessed using proprietary radioimmunoassay kits.

In this study, endometrial explants were essentially unchanged at the end of the treatment period for the intact controls. For other groups there was clear evidence of atrophy. Serum E₂ was only detected in the intact controls, whilst serum P₄ was significantly lower in all ovariectomised animals compared with controls. Effects of treatment with Danazol or Ethynyl E₂ and norgestrel were observed for serum progesterone and cytosolic oestrogen and progesterone receptor levels. It was considered that although none of the treatment regimens showed a gross effect upon endometrial tissue morphology, the differences in cytosolic receptor levels were evidence of a direct effect at a cellular level.

Mouse model:

- **Explant diameter:** measured to the nearest 0.1 mm using callipers. A mean of the three measurements per mouse was calculated, and this mean value was used in group mean calculations.
- **Histological appearance of explant endometrium:** the appearance of the endometrial glands and stroma was assessed. No between-group comparisons were, however, reported.

Satisfactory surgical induction of endometriosis was possible by this method, although greater surgical dexterity was necessary than for the rat; even then, a death rate of approximately 10% was experienced. Growth rates of the explants were similar to those of the rat. Following ovariectomy, regression of the sites was apparent in the untreated controls. Treatment with oestrone, however, resulted in endometriotic growth greater than that of the intact animal.

RODENT VAGINAL ASSAYS

Since the early part of this century, the potential for chemicals to modulate the structure and function of the rodent vagina has been recognised. These methods have generally provided effective, reliable and easy-to-record endpoints, and both rat and mouse models have been established. Rat models are reviewed in the Section Rat Vaginal Models. Those for mice are presented below.

MOUSE MODELS

In a 1954 paper, Biggers and Claringbold reported on the classification of vaginal smears in the mouse and detailed the changes seen in response to oestradiol treatment.

The data derived were statistically analysed in detail to test the validity of the scoring systems used.

METHOD

Two inbred strains of mice, the C57 and the CBA, together with their reciprocal F₁ hybrids, were used. Animals were ovariectomised before the study. Animals were assigned to treatment groups and subjected to the following treatment regimens:

Oestradiol was administered to the mice as two daily doses by either the subcutaneous or intravaginal route. For the subcutaneously injected animals, smears were prepared at 56, 78 and 80 hours after the first dose whilst intravaginally dosed animals were examined after 48, 56 and 72 hours.

Both the standard quantal (all or nothing) scoring system and a four-fold criteria (denoted as either 0, x, y or 1) were used, using the following criteria:

Normal quantal score criteria	Four-fold score criteria
- Leukocytes, mucin, nucleated or cornified cells	0 leukocytes, mucin and a few cells only x leukocytes, mucin, many nucleated or cornified cells
+ Nucleated and/or cornified cells only	y nucleated or nucleated and cornified cells only 1 cornified cells only

Data obtained from the mouse vaginal smear examinations were statistically treated and the results compared for the two recording systems. For each route of administration, the results from the two inbred lines and the two hybrids were analysed separately, using groups of 12 animals, with nine dosage groups.

A statistical discriminate analysis technique was used to analyse the additive four-fold scores. The total response for each group was calculated from the maximum scores achieved by individual animals, using the equation:

$$ax + by + c$$

where *a* is the number responding at level *x*, *b* the number showing level *y* responses, and *c* the number responding to *I*. The response for each animal was scored on the basis of the highest achieved score (from the sequence: $0 < x < y < 1$) from all the smear examinations. Data were analysed for variance.

Results from the quantal scores (*i.e.* + or -, in which *x* and *y* were also scored as 0 and 1, respectively) were analysed for variance and covariance. Data were also analysed using other nominal values for *x* and *y* (*e.g.* *x* = 0.33 and *y* = 0.67).

SUBSEQUENT DEVELOPMENT

Vaginal opening/smearing

Ng *et al.* (1994) included an assessment of mouse vaginal responses using a smear assessment in their work on yuehchukene.

Randomly bred, ovariectomised QS mice were primed with subcutaneous oestradiol one week after surgery. Animals could be maintained on single doses of oestradiol every two weeks until required. For the study, animals were given intravaginal doses of vehicle alone, yuehchukene or oestradiol for two days. Smears were taken on day 4 of the study, stained with methylene blue and scored positive if they contained nucleated epithelial and/or cornified cells but no leukocytes.

Vaginal morphology

Martin and Claringbold (1958) reported on the use of a model involving histopathological examination of the vaginas of ovariectomised mice.

Randomly bred ovariectomised albino mice were primed with a subcutaneous injection of oestrone two weeks before commencement of the study and randomly assigned to treatment groups. Group sizes varied between eight and 48 animals. The test material was administered intravaginally 24 hours before humane killing. Chemicals studied comprised oestrone and the combination of oestrone and oestradiol (various dosages were used to derive dose-response curves). Seven hours before humane killing, animals received a subcutaneous injection of colchicine. Subsequently the vaginas were removed, fixed, and Heidenhain's haematoxylin-stained sections examined by light microscopy. Five fields were randomly selected and the total number of arrested mitoses were counted in each field and added together to give a total score for each animal. A transformation, $Y = \log_{10}(Z + 2)$, was applied, where *Z* is the number of mitoses per field followed by analysis of variance.

Martin and Claringbold (1960) reported on further work using a very similar model to that described above. The first part of this paper investigated various dosage regimens and times of humane killing with oestrone as the oestrogenic agent. In most experiments the administration regimen was, however, as described in the above paper. The effects of treatment with a series of other test chemicals were investigated for a range of dosages; these included oestradiol, progesterone, cortisone, cortisol,

dienoestrol, hexoestrol, diethylstilboestrol and testosterone. The effects of various routes and dosages, and the timing of colchicine administration, were also investigated.

Additional details were presented on the method of ovariectomy of the animals. The ovaries were removed by cutting the uterine horn slightly below the tip with the scissors used to make the skin and body wall incisions. Wounds were closed by gentle pressure from fingers, and penicillin and streptomycin solution applied. No sutures were used; animals were held under a strong light source for 18 hours to promote wound drying. Death rates were noted as being below 2-3 %.

Endpoints investigated included mitotic index as performed above, plus assessment of five fields from the sections examined for epithelial thickness using an eye-piece micrometer, and expressing the results in arbitrary units. The scores from each field for an animal were totalled to give a value for use in subsequent calculations. An increase in thickness at 24 hours after the treatment stage would be due to hypertrophy rather than hyperplasia, because little or no cell division would have occurred before 16 hours and subsequent activity is arrested by the colchicine treatment.

Vaginal weight

The vaginotropic aspects of work by **Folman and Pope (1966)** are reported here; concurrent uterotrophic investigations were reviewed in Section 0 above. In summary, the experimental procedure involved the daily subcutaneous administration of test chemicals to female albino BSVS mice for a three-day period. Seventy to 74 hours after the first administration, animals were weighed and vaginas removed and left for one hour in an atmosphere saturated with water before weighing.

Vaginal triphenyltetrazolium chloride assay

Martin (1960) published a paper on a mouse vaginal triphenyltetrazolium chloride assay model for the assay of oestrogens. 2-3-5-triphenyltetrazolium chloride is capable of interaction with intracellular reductases, producing a stable, water-insoluble deep red pigment, formazan, which can be extracted from tissues and measured colourimetrically. The method was developed in the belief it might act as an index of early respiratory changes following oestrogenic stimulation.

Adult, albino Sydney white mice were used seven to ten days after ovariectomy, with no prior oestrone priming. In most experiments mice received a single intravaginal administration of the test chemical and were killed 24 hours later. Intravaginal triphenyltetrazolium was administered 30 minutes before humane killing. Vaginas were dissected, washed in distilled water, dried and placed in ethanol-tetrachlor-ethylene. The amount of pigment produced was then measured colourimetrically. In a series of early experiments, various dosage regimens and schedules for triphenyltetrazolium addition were investigated. In addition, in some instances vaginal weight was also recorded following placement on filter paper to assist in removal of excess fluid.

Potency relative to oestrone was assessed for 17β -oestradiol and diethylstilboestrol. For these studies, groups of five to seven animals received the appropriate test material at one of four to six dosages. Specificity for oestrogenic stimulation was assessed by investigating the response to progesterone, testosterone and cortisol treatment in groups of six mice given one of two dosages of a chemical.

ENDPOINTS/DATA INTERPRETATION

Biggers and Claringbold (1954); Ng *et al.* (1994):

- **Induction of oestrus:** assessed by examination of vaginal smears.

Biggers and Claringbold reported that the statistical analyses indicated that the response data were strictly quantal; this applied to all the lines studied and both routes of administration. On this basis the authors proposed that smears classified as denoting proestrus should be considered positive and that the absence or presence of leukocytes should be a critical principle for scoring as positive or negative. Overall, they suggested that the correct method for analysis of vaginal response is to use a strict binomial distribution and not an arbitrary, assumed additive score.

In the study by Ng *et al.*, a dosage-related response to oestradiol treatment was recorded between dosages of 5 and 500 pg. A similar dose-response curve was derived for co-administration with 5 ng of yuehchukene. A stimulatory affect of yuehchukene was apparent at a dosage of 50 ng whilst at 500 ng it exerted an inhibitory response to oestradiol treatment at higher levels; it was considered that this indicated that the chemical was a mixed agonist/antagonist.

Martin and Claringbold (1958 and 1960):

- **Vaginal mitotic rate:** total number of arrested mitoses counted by light microscopic examination.
- **Vaginal epithelium thickness:** measured using an eyepiece micrometer, expressed in arbitrary units (1960 paper only).

In the 1958 paper, it was reported that oestrone showed a linear response curve from 0.5×10^{-5} to 8×10^{-5} μg when response was plotted against log dose, with a maximal response at the latter dosage. The slope derived for oestradiol was similar and a relative potency of 1.68 was found. Martin and Claringbold commented that this method appeared to be ten times as sensitive and four times as precise as the standard intravaginal assay.

In the 1960 paper, it was shown that mitosis and thickening are not significantly affected during the first 16 hours following treatment. This was followed by a transitory increase in mitotic rate that appeared to be linear with dosage, with a maximum at 24 hours. A similar increase without the subsequent decrease was seen for epithelial thickness. On this basis, it was concluded that the 24 hour point offered the highest sensitivity. In subsequent experiments they found that mitotic rate appeared to be subject to depression at very high dosages. The aetiology was not clarified. Systemic administration of colchicine was found to decrease mitotic rate and epithelial thickness; the slopes of the respective dose-response lines were unaffected. Local administration affected only mitotic rates by arresting cells at metaphase. Systemic administration of colchicine was, however, selected for subsequent work because of its greater convenience. Joint administration of oestrone with androgen, progesterone, cortisol or cortisone showed no effect upon either endpoint studied. Administration of progesterone, testosterone or cortisol, subcutaneously or intravaginally, at very high levels in combination with a single intravaginal application of oestrone was found not to inhibit cell growth or division. Testosterone was found to be mitogenic by both routes, whilst cortisol and, to a lesser extent, progesterone were active locally. The authors stressed that this need not imply that these chemicals were oestrogenic, because metabolism to oestrogens or oestrogen contamination at one part in ten

million would have been sufficient to account for the level of activity observed. Potencies of oestriol, dienioestrol, hexoestrol and diethylstilboestrol were found to be similar to that of oestrone.

The authors claimed that this model possesses certain advantages over the Allen-Doisy technique. Thus, although it involves humane killing of animals, the duration is shorter, sensitivity is much higher, and the precision is greater than in the standard assay. The assay permits time course investigations and the clarification of primary and secondary oestrogenic modulation.

Folman and Pope (1966):

- **Vaginal weight:** as absolute wet weight.

The weak oestrogens coumesterol, genistein and dimethylstilboestrol, and the progestins norethisterone and megestrol acetate, were weakly stimulatory, although values for megestrol acetate were not significantly different from the controls statistically. However, it was observed that the percentage increase in vaginal weights was less than could be elicited in the uterus and that the efficacy for the two endpoints varied between chemicals.

Martin (1960):

- **Vaginal weight:** as absolute wet weight.
- **Vaginal triphenyltetrazolium chloride:** conversion assessed colourimetrically.

Experiments on the optimal times for killing after dosing with the oestrogenic chemical, and of triphenyltetrazolium administration, showed them to be 24 hours and 30 minutes, respectively. Organ weight data proved of relatively little value.

Potency relative to oestrone was assessed for 17β -oestradiol and diethylstilboestrol; values derived were 1.4 and 2.8, respectively, which for oestradiol was recorded by the author as being in close agreement with those derived previously using mitosis, epithelial thickness or vaginal cornification endpoints. For diethylstilboestrol, potency by this method was higher than previously recorded; the reason was not identified. Testosterone, progesterone and cortisol treatment did not affect the reduction of triphenyltetrazolium even at levels 1000 times that of oestrone.

In summary, the author commented that this method gave similar sensitivity and accuracy to the mitosis and epithelial thickness assays and had the added advantage of extreme simplicity.

RODENT MAMMARY GLAND

In a recent study, Brown and Lamartinière (1995) demonstrated a range of effects by known endocrinologically active chemicals on the rat mammary gland. The chemicals used were selected on the basis of literature reports of their endocrine activities. They comprised: oestrogenically active diethylstilbestrol (DES), genistein, Arochlor 1221 and *o,p'*-DDT; and the anti-oestrogen 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Additional groups received either Arochlor 1254 (non-oestrogenic but greater mammalian toxicity than Arochlor 1221) or sesame oil.

METHOD

The test model was the female Sprague-Dawley CD rat. Animals were obtained following weaning and maintained under standard housing conditions. Six animals per group were treated once daily on four occasions, either by subcutaneous injection or oral gavage, according to the following regimen:

Treatment	Route	Dosage	Treatment day (post partum)
Sesame oil	Subcutaneous	200 µL	23, 25, 27, 29
DES	Subcutaneous	50 ng/g	23, 25, 27, 29
Genistein	Subcutaneous	50 µg/g	23, 25, 27, 29
<i>o,p'</i> -DDT	Subcutaneous	50 µg/g	23, 25, 27, 29
Sesame oil	oral gavage	200 µL	25, 27, 29, 31
Arochlor 1221	oral gavage	25 µg/g	25, 27, 29, 31
Arochlor 1254	oral gavage	25 µg/g	25, 27, 29, 31
TCDD	oral gavage	25 ng/g	25, 27, 29, 31

The dosage regimen was derived from literature review and the authors' unpublished data. Animals were killed 18 hours after the last administration and the abdominal mammary glands dissected. One gland was used to prepare a whole mount, whilst the other was processed to the block stage and sections were prepared as detailed below. Bodyweight and uterine and ovary weights were recorded to compare with the results from the mammary gland endpoints. Data were subject to statistical analysis by Student's t-test (two-tailed).

Whole mount:

The whole of one abdominal mammary gland was spread on a slide, fixed with 10% neutral buffered formaldehyde, defatted and stained in alum carmine (2 g/L). The stained gland was dehydrated, cleared and compressed under a secured second slide for 24 hours, then allowed to expand before permanent mounting under a coverslip. Whole mounts were examined under light microscopy at 40× and 100× magnification and scored for numbers of alveolar buds and lobules. The outer portion of the entire gland was evaluated and terminal duct structure assessed. The size of the mammary gland was also measured.

Mammary gland sections:

After dissection, one gland was fixed in formalin and processed for paraffin embedding within 24 hours. Sections were cut at 5 µm, stained and mounted. Sections were deparaffinised, endogenous peroxide activity was quenched, and non-specific IgG binding suppressed. Tissues were incubated with proliferating cell nuclear antigen (PCNA), biotinylated horse anti-mouse secondary antibody, and avidin-

biotin-bound peroxidase. Colour was developed by 3,3'-diaminobenzidine tetrahydrochloride and cells were counterstained with Gills No. 2 haematoxylin. Cell cycles were assessed using an image analyser for immunocytochemical staining patterns of PCNA, and a proliferative index was derived. In addition, cells in S-phase were characterised by their uniform dark-brown to black nuclear staining and a labelling index was derived.

ENDPOINTS/DATA INTERPRETATION

The following endpoints were used:

- **Mammary gland size** (mm²): determined using an image analyser system coupled with video printer and sonic digitiser. The system used in-house developed software. Calibration was achieved using a micrometer photographed with the gland.
- **Terminal ductal structure**: classified as terminal end buds, terminal ducts, alveolar buds or lobules type 1, dependent on diameter and number of lobules. The detailed criteria used were based upon work by Russo and Russo (1978).
- **Proliferative index**: percentage of epithelial cells in the active cell cycle (*i.e.* G₁ + S + G₂ + M) divided by the total number of epithelial cells counted.
- **Labelling index**: number of epithelial nuclei in S-phase divided by total number of epithelial cells counted, expressed as a percentage.

On the basis of the results, the authors considered that they had shown that acute exposure of rats to DES, genistein and *o,p'*-DDT during the pubertal period increased mammary cell proliferation and enhanced gland differentiation whilst TCDD inhibited both proliferation and development. Effects of Arochlor 1221 and Arochlor 1254 were slight but not statistically significant. This was attributed to an inadequate dose or weak oestrogenic properties. It was important to consider the window of exposure and the developmental maturation of the mammary gland.

MOUSE PROSTATE MODELS

THE “neoDES” MOUSE MODEL

The involvement of sex hormones in the development and functioning of the rodent prostate has been widely studied. A number of workers have used the sensitivity of the mouse prostate to oestrogenic chemicals to develop related model systems.

The animal model on which these systems are based is the “neoDES” mouse described by Pylkkänen *et al.* (1991). This model was developed on the basis of the authors’ belief that exposure of experimental animals to oestrogens during the fetal or neonatal period would lead to permanent inhibition of the androgen-dependent growth and function of the prostate. The 1991 study was undertaken to investigate changes in the oestrogen responses and the composition of oestrogen-dependent cells preceding or associated with altered prostatic growth in developmentally oestrogenised mice.

METHOD

Outbred Han:NMRI mice were weaned at 22 days of age and housed under standard conditions. Neonatal oestrogenisation was achieved by injecting neonatal mice with diethylstilboestrol (DES) subcutaneously. Initially a number of age ranges and dosages were investigated to determine the most effective. These were days 1-5: 2 µg/pup/day; days 4-6: 3 µg/pup/day; days 7-9: 4 µg/pup/day. At the age of nine to 10 weeks, animals were decapitated and the weights of the prostatic lobes immediately recorded following removal of seminal vesicle secretions.

In further experiments, the neonatal oestrogenisation consisted of three injections of DES on days 1-3 or five injections on days 1-5 of age. Controls received corn oil alone. At two months of age, five controls and five treated animals were castrated and implanted with pellets containing 17β-oestradiol. Animals were killed at various ages thereafter.

At necropsy, the whole urethro-prostatic complex was preserved in Bouin’s fixative. Specimens were embedded in paraffin wax and serial sections were prepared and stained with haematoxylin and eosin. For each animal, the largest representative section of ventral lobes and coagulating glands was selected. Morphometric analysis was achieved by visual observation using a microscope fitted with a Weibels multipurpose screen with 21 test lines and 42 test points. From eight fields of both ventral lobes and coagulating gland, the relative volumes of glandular epithelium, glandular lumen and stroma were counted. In addition, five control and five treated animals were killed by decapitation and the whole urethro-prostatic complex removed and frozen in liquid nitrogen. Frozen tissues were mounted in Tissue-Tek II O.C.T Compound (Miles Scientific, Naperville, Illinois), sectioned on pre-cooled cryostats and thaw-mounted onto tissue adhesive-treated slides. Fixation and staining was achieved using a commercial antibody kit. After one hour’s incubation, oestrogen-positive cells were identified using adjacent sections stained with haematoxylin and eosin or Weigert and van Gieson staining.

Other animals were left for ten days after castration before receiving a single subcutaneous injections of either 5α-dihydrotestosterone or 17β-oestradiol alone or both in combination. Animals were killed six hours after receiving an intraperitoneal injection of ³H-thymidine and 48 hours after the hormonal treatment. Tissue sections were prepared for autoradiography by coating with nuclear emulsion and exposing for 14 days at -15°C. Sections were subsequently developed and stained with haematoxylin and

eosin. Relative volumes of epithelium and stroma were calculated as above and labelled nuclei in each tissue compartment were then counted from the same test areas; eight fields per sample were examined.

Weight data and prostate morphometry volume densities of acinar epithelium, lumen and intracinar tissue were analysed by a one-way analysis of variance followed, where appropriate, by Student's t-test with Bonferroni correction. Analysis of thymidine labelling data required one-way analysis of variance with square root transformation because of the high variances found.

SUBSEQUENT DEVELOPMENT

Pylkkänen *et al.* (1993) reported further work studying the long-term changes in the neoDES mouse model. Mice were treated with either DES or 17 β -oestradiol daily for the first three days postnatally. These neonatally treated mice were allowed to age for a year or, for a small number of animals, up to 18 months before examination. The responses seen were compared with those for control mice or those exposed either to DES prenatally on days 13 to 15 of gestation (termed "preDES" animals) or to an anti-androgen (cyproterone acetate) on days 1 to 3 postnatally. The effect of secondary hormonal exposure was investigated by implanting pellets of DHT and/or 17 β -oestradiol into control and neoDES mice at nine months of age and performing histological examinations once the animals reached one year of age. Endpoints determined included weight of the prostate, seminal vesicles and testes, severity of prostatic dysplasia assessed by histopathological examination of coded slides (*i.e.* "blind" reading), and assessment of c-myc RNA expression of the ventral and dorsolateral lobes of the prostate. For this latter endpoint, tissues were frozen in liquid nitrogen at necropsy, homogenised, and an RNA pellet produced by centrifugation at 35,000 g for 20 hours at 20°C. After dissolving in Tris-EDTA and extraction of the proteins, the RNA was precipitated and, after washing and re-suspension, was subject to electrophoresis on agarose gel, re-suspended and transferred to a GeneScreen membrane. DNA probe c-myc was labelled by a random oligonucleotide priming method using a [³²P]-labelled probe. The autoradiograms from the exposures were scanned using a densitometer to quantify the relative intensities of hybridisation with the c-myc proto-oncogene probe. Organ weights were subject to statistical analysis by one-way analysis of variance and Student's t-test with Bonferroni correction.

Mäkelä *et al.* (1995 a, b) reported the use of the neoDES mouse model to test the oestrogenicity and anti-oestrogenicity of dietary soya and two structurally different phytoestrogens known to be present in soya, coumesterol and diadzein. These papers report studies in which adult neoDES mice were fed soya-containing diet or given coumesterol or diadzein. Endpoints investigated included organ weight, histopathological appearance and c-fos expression; c-fos was measured using a similar procedure to that for c-myc (see above).

ENDPOINTS/DATA INTERPRETATION

Pylkkänen *et al.* (1991):

- **Prostate weights:** recorded at necropsy.
- **Thymidine incorporation:** number of labelled nuclei per unit of tissue volume calculated for autoradiographic sections from stromal and epithelial area and nuclei count data.
- **Oestrogen receptor incidence and location:** identified by immunocytochemistry and microscopic evaluation.

It was found that neonatal exposure to DES caused a dose-dependent and time of treatment-dependent reduction in size of prostatic lobes and seminal vesicles by nine to 10 weeks of age. The critical time for persistent effects was days 1-6 postnatal.

Morphometric analysis of the ventral lobes and coagulating glands showed a decrease in the volume density of glandular lumina and an increase in that of stroma. Epithelial hyperplasia in the posterior periurethral collecting duct and coagulating glands and inflammatory cells in the prostate were observed in hormonally treated animals at two months of age. By nine months of age, the difference in prostate size between control and treated animals was still evident and dysplastic changes were apparent in the epithelium of the coagulating glands, periurethral collecting ducts, and periurethral collecting ducts and glands of the hormonally treated animals but not in those of the controls.

Measurement of tritiated-thymidine incorporation revealed a marked increase in response to DHT treatment of the animals neonatally exposed to DES. Secondary treatment of castrated control and neonatally DES-treated animals with oestradiol did not significantly affect labelling of the epithelium or stromal cells in the coagulating gland or ventral prostate lobe. In contrast, co-treatment with DHT and oestradiol abolished the DHT-induced increase in cell-labelling.

Early exposure to DES resulted in a permanent modification of the response of the male genital tract to secondary oestrogen treatment. Treating castrated mice that had received DES for the first three days of life with an oestrogen pellet for seven days induced extensive squamous epithelia metaplasia in the periurethral collecting ducts and proximal coagulating glands. The immunocytochemical studies demonstrated that the oestrogen receptors were localised in the nuclei of the stromal cells surrounding the prostatic collecting ducts, ejaculating ducts and periurethral glands inside the muscular layer of the urethra; the appearance of neonatally oestrogenised animals treated with DES or DHT was, however, similar to that of the controls.

Pylkkänen *et al.* (1993):

- **Prostate, seminal vesicles and testes weights:** recorded at necropsy, expressed relative to bodyweight.
- **Prostatic dysplasia:** by histopathological examination.
- **c-myc expression:** of the ventral and dorsolateral lobes of the prostate using autoradiographic techniques.

Analysis of organ weights in one-year-old animals showed reduced bodyweight-relative ventral prostate, coagulating gland and seminal vesicle weights in the neoDES mice when compared with controls. In contrast, cyproterone acetate treatment affected principally the seminal vesicle weight. Histopathologically, dysplasia of the prostate was found in mice given DES neonatally (*i.e.* neoDES) and to a lesser extent in those given oestradiol neonatally; the severity increased with increasing age. The prostate of prenatally oestrogenised animals was, however, unaffected. Secondary hormonal treatment at nine months of age augmented the dysplasia seen in the neoDES animals. Analysis of c-myc expression showed a consistent increase in all prostatic regions of the neoDES animals. This was an organ-specific response in the neoDES mice because a similar increase was not observed in their other organs such as the kidney, liver or testis.

Mäkelä *et al.* (1995 a, b):

- **Prostate, seminal vesicles and testes weights:** recorded at necropsy, expressed relative to bodyweight.
- **Prostatic dysplasia:** by histopathological examination.
- **c-fos expression:** of the prostate using autoradiographic techniques.

During short-term studies, feeding of soya-containing diets or coumesterol treatment to adult neoDES mice for up to ten days had no influence on the expression of c-fos nor squamous metaplasia of the collecting ducts as seen in animals given secondary oestradiol treatment. In the absence of any secondary oestradiol treatment, these treatment regimens did not elicit any metaplastic responses in their own right. In contrast, feeding dams and their pups soya from fertilisation onwards, and subjecting the male pups to neonatal DES treatment (*i.e.* to induce the neoDES state), reduced the prostatic growth inhibition and appeared to delay the dysplastic changes in the prostate normally caused by the DES treatment. The authors considered this to suggest that the anti-oestrogenic action of the soya could be exerted during the neonatal period.

MOUSE PRENATAL EXPOSURE/ADULT PROSTATE MODEL

This model was developed by vom Saal and colleagues to investigate the role of oestradiol in prostate development. In earlier studies, male mice positioned *in utero* between two females had been found to be exposed to increased levels of serum oestradiol when compared with males positioned between two males (Even *et al.*, 1992). This increase was associated with significant enlargement of the prostate and changes in behaviour in adulthood. Whilst androgen is the primary mediator of prostate differentiation, the authors proposed a modulating role for oestrogen in prostate development based on these observations.

The model was used in a series of experiments by vom Saal *et al.* (1997) to examine the relationship between natural and synthetic oestrogen levels during fetal life, and endpoints including prostate size and prostatic androgen receptor number in fetal and adult life.

METHODS

Outbred CF-1 mice were used in the study and were housed in standard polypropylene mouse cages on corn cob bedding and fed Purina lab chow. Rooms were kept at 23°C with a 12-hour light (on at 24:00) and 12-hour dark cycle.

Assessment of oestradiol levels and changes in prostate structure in male fetuses exposed to supplemental oestradiol

On gestation day 13, pregnant females (six per group, time-mated, confirmed by presence of copulatory plug) were implanted subcutaneously with a 10 mm-long Silastic capsule containing one of five doses of oestradiol (0, 25, 100, 200 or 300 µg) dissolved in 20 µL of sesame oil. On gestation day 18, the animals were killed by CO₂ asphyxiation and cervical dislocation, and blood was collected from all male fetuses positioned *in utero* between a male and a female fetus. Total serum oestradiol and testosterone levels were measured by radioimmunoassay for each fetus, whilst levels of free oestradiol were measured using pooled sera from each litter (n = 6-8 per group) by centrifugal ultrafiltration dialysis. The concentration of free serum oestradiol was calculated by multiplying the percentages of free oestradiol by the total serum oestradiol concentration. One male fetus positioned between a male and a female was selected at random from each litter. The urogenital sinus and developing prostate were removed, fixed in Bouin's solution, sectioned and examined by computer-assisted three-dimensional reconstruction. Outlines of the prostatic buds in the dorsal, lateral and ventral budding lines were used to calculate the number of prostatic buds, the length of the line of buds along the urogenital sinus, and the total area and mean cross-sectional area for buds in a budding line. Similar measurements were made for the utriculus (remnant of the Müllerian ducts which is enclosed within the adult prostate).

Assessment of changes in prostate weight and prostatic androgen receptors in adult male mice following prenatal oestradiol treatment

Pregnant female mice implanted with capsules containing different amounts of oestradiol (see above) were humanely killed on gestation day 19. Male offspring lying between a male and a female were identified and reared with litter mates by foster dams. In addition to a zero dose control group (sham control), a second control group consisted of males carried by pregnant females that had not been handled throughout pregnancy. Males were weaned at 23 days old and housed two to four (from the same litter) to a cage until seven months old. At this time, one randomly selected litter mate was removed and housed

alone to control for litter effects. To control for possible effects of prenatal treatment on functioning of the brain-pituitary-testicular axis, these males were castrated under anaesthesia one week after being individually housed and implanted s.c. with a 10 mm Silastic capsule containing 500 µg testosterone dissolved in 20 µL sesame oil. Males were killed three weeks later, bodyweight was recorded, and the prostate removed, weighed, frozen in liquid nitrogen and stored at -70°C. To measure the level of cytosolic prostatic androgen receptors, prostate cytosols were prepared in Tris-HCl/EDTA/NaMoO₄/DTT/phenylsulfonyl fluoride/glycerol at 1-2 mg protein/mL. Dextran-coated charcoal was used to strip endogenous androgens. Cytosol (100 µL) was then added immediately to 250 µL hydroxyapatite (60%) in Tris-HCl (50 mM). The mix was made up to 20 nM with [³H]dihydrotestosterone (DHT) with or without a 100-fold excess of non-radioactive DHT in a separate tube to measure non-specific binding. Following overnight incubation at 4°C, specific binding of DHT to the androgen receptors was determined and normalised to cytosol protein or total DNA. Using high salt extracts, receptors occupied by endogenous ligand ("nuclear" receptors) were determined but values were less than 10% of cytosolic receptors.

Assessment of changes in prostate weight in adult male mice following prenatal diethylstilboestrol treatment

Diethylstilboestrol (DES) was dissolved in tocopherol-stripped corn oil so that 30 µL contained concentrations of 0, 0.002, 0.02, 0.2, 2.0, 20 and 200 ng/g bodyweight. These concentrations were fed to pregnant mice (n = 6-8 per group) once daily by electronic micropipette (to reduce the stress of gavage, which might interfere with sexual differentiation) from gestation days 11 to 17. An additional control group of females (n = 7) remained unhandled throughout pregnancy. Females were allowed to deliver and nurse their own offspring, and male offspring were weaned on day 23 when two to four males from the same litter were housed together. At seven months old, one randomly selected male from each litter (n = 6-8 per group) was removed and housed individually. One month later, males were killed and body and prostate weights were recorded.

Statistical analysis

The Statistical Analysis System GLM procedure was used. Planned comparisons were made using the Least Squares means test, rejecting the null hypothesis at $p < 0.05$. Pearson's correlation coefficient was used to determine correlations between body and prostate weight in adulthood. Prostate weight was also analysed by covariance (ANCOVA) and adjusted for bodyweight to determine if this accounted for a significant component of the prostate weight variance. If there was no correlation on the basis of ANCOVA analysis, prostate weight was reanalysed by ANOVA.

ENDPOINTS/DATA INTERPRETATION

- **Prostate weights:** recorded at necropsy.
- **Fetal prostate analysis:** by three-dimensional computer-assisted reconstruction. Endpoints measured included number of buds, total area of buds, length and mean area.
- **Total serum oestradiol and testosterone analysis:** concentrations measured by radioimmunoassay.
- **Free serum oestradiol analysis:** measured by centrifugal ultrafiltration dialysis.

- **Prostatic androgen receptors:** specific binding to androgen receptors determined using [³H]dihydrotestosterone; non-specific binding assessed using an excess of non-radiolabelled DHT.

Assessment of oestradiol levels and changes in prostate structure in male fetuses exposed to supplemental oestradiol

Capsule doses of oestradiol increased both total and free serum oestradiol levels in male fetuses relative to control fetuses. In the highest dose group (300 µg), there was a significant increase in total serum testosterone. Oestradiol treatment significantly increased the number of prostatic buds and the length of the line of prostatic glandular buds along the urogenital sinus relative to controls. Urogenital sinus length occupied by the utriculus increased significantly in oestradiol-treated males, and there was a significant decrease in mean cross-sectional area of the lumen of the urethra in the region of the prostate.

Assessment of changes in prostate weight and prostatic androgen receptors in adult male mice following prenatal oestradiol treatment

The two groups of control animals did not differ significantly from each other for any measure and so were combined as one group. Prenatal treatment with oestradiol produced a significant increase (27%) in adult male prostate weight compared with the control group at the 25 µg dose. In contrast, the highest capsule dose resulted in a significant decrease in adult prostate weight relative to controls. Prenatal oestradiol exposure had no significant effect on adult body weight. The lowest dose group (25 µg) had a six-fold increase in the total number of androgen receptors per prostate, a two-fold increase in numbers of androgen receptors per cell, and a 40% increase in DNA per prostate, demonstrating prostatic hyperplasia (all observations $p < 0.05$).

Assessment of changes in prostate weight in adult male mice following prenatal diethylstilboestrol treatment

The two groups of control animals did not differ significantly from each other for any measure and so were combined as one group. After correction for bodyweight by ANCOVA (bodyweight accounted for a significant proportion of variance in prostate weight), prostate weights in males in the 0.02, 0.2 or 2ng/g DES dose groups were significantly increased relative to controls, whilst prostate weights in males in the 200 ng/g group were significantly smaller than in the controls and other treatment groups. Body weight differed significantly as a function of dose of DES ($p < 0.001$), with significantly lower values in the 200 ng/g dose group relative to controls.

RODENT BEHAVIOURAL MODIFICATION

vom Saal *et al.* (1995) reported on the use of a mouse model in which adult behaviour was monitored for effects resulting from modulation of oestrogenic exposure during the fetal period.

The authors monitored the urine-marking behaviour of adult male mice that had been exposed *in utero* to *o,p'*-dichlorodiphenyltrichloroethane (*o,p'*-DDT), methoxychlor or diethylstilboestrol (DES). Such marking activity is common behaviour in rodents, and one that has important consequences for their social interactions and reproductive behaviour.

METHOD

CF1 mice were time mated and randomly assigned to one of 18 groups (six to 10 per group) on day 11 of pregnancy. Unhandled/undosed and handled/vehicle control groups were included. On days 11 to 17 of pregnancy, treated animals received the appropriate dosage of either *o,p'*-DDT, methoxychlor or DES by electronic micropipette at a dose volume of 30 µL. Animals readily consumed the dose in a corn oil vehicle supplied in this manner, thus preventing any stress on the animals from oral gavage. Dosages used ranged from 0.001 to 10.0 µg/day DES, 1-5000 µg/day *o,p'*-DDT and 1 to 5000 µg/day for methoxychlor. Once littered, only two males from each litter were used for postnatal effect assessment. At 60 days of age, these F₁ animals were individually housed for four weeks to eliminate any effects of previous housing with other males and then subject to marking assessment.

Animals were monitored for one hour in clean cages (30 × 30 × 10 cm) divided into two chambers by a metal mesh barrier. The flow of the cage was covered with a piece of filter paper. Test males were placed in the cage with a 90-day-old, unhandled female in the other compartment. At the end of the period, the filter paper was removed and urine marks deposited by the male counted under UV-light.

ENDPOINTS/DATA INTERPRETATION

- **Urine marks:** counted for one hour collection period using UV-light.

Except for females treated with 10 or 100 µg/day, DES animals delivered normally. Animals in these dosage groups either delivered late or lost the litter. Assessment of urine marking behaviour showed an increase in males given the lowest prenatal dose of DES. In the 10µg/day group, the levels were significantly lower than at the low dosage. This U-shaped response curve is typical of oestrogenic developmental effects. In contrast, the other test chemical elicited a normal dosage-related response pattern. Modifications to the behaviour patterns of animals, particularly ones that play a vital part in the reproductive success of male mice, are of potentially profound importance.

ANDROGEN RELATED

CHICKEN COCKSCOMB TOPICAL BIOASSAY

Introduction

The use of the chicken cockscomb assay to quantify anti-androgenic activity was reported in 1958 by Dorfman. In this paper the inhibitory effects of two chemicals on the growth of young chickens was assessed in terms of combweight-bodyweight ratio.

Method

Two-day-old white Leghorn cockerels were assigned to treatment groups of between 12 and 44 and subcutaneously injected with testosterone enanthate in sesame oil. On this day and for the next six days, test material (either Northisterone or 2-acetyl-7-oxo-1,2,3,4,4a,4b,5,6,7,9,10,10a-dodecahydrophenanthrene) was applied dermally to the comb once daily at various dosages. On the day after the last administration, the chickens were killed and the bodyweight and combweight determined.

Subsequent development

Hennessey *et al.* (1986) reported the use of a modified cockscomb assay to investigate differences in anti-androgenic potency of spiro lactone and cimetidine. Differences from the study by Dorfman included the use of one-day-old Dekalb XL cockerels which were given daily subcutaneous injections of dihydrotestosterone. The chemicals tested for androgenicity were applied twice daily until the animals were killed at 15 days of age. A group size of 14-18 birds was used. Statistical analysis was by ANOVA, with Scheffé's test used to determine the doses producing significant or maximal suppression of comb growth. Student's t-test was used for intergroup comparisons.

Endpoints/data interpretation

Comb ratio: calculated as mg comb per gram of bodyweight.

In the study by Dorfman, both chemicals inhibited the stimulatory action of testosterone on comb growth. In the later study by Hennessey *et al.*, dosage-related anti-androgenicity was seen for both chemicals tested and it was possible to distinguish differences in their potencies. There are several benefits for this system: very simple methodology; use of inexpensive, easy to maintain animals; and the assay allowed study of local action uncomplicated by systemic metabolism (*i.e.* topical application).

QUAIL UROPYGIAL GLAND ASSAY

Introduction

Daniel *et al.* (1986) demonstrated the use of the quail uropygial gland as a marker for anti-androgenic activity. The test is based upon the uropygial gland of the male Japanese quail (which is the counterpart of the mammalian sebaceous gland) responding to androgens by increasing cellular concentrations of androgen receptors (AR) and of dodecane-2,3-diol (C12-diol) in the gland's secretory products. The level of testosterone in animals is controlled by the use of castrated birds, and the levels of AR and C12-diol are compared for animals receiving either testosterone alone or in combination with the putative anti-androgenic agent.

Method

Male Japanese quail were castrated before study. Both single and multiple dose regimens and dermal and subdermal administration were studied.

Single dose study: Groups of nine birds were assigned to the following treatment regimens:

Group	Testosterone injection	Test material injection
1	No	No
2	Yes	No
3	Yes	Low
4	Yes	Intermediate
5	Yes	High

The appropriate dose of test material was injected subdermally close to the uropygial gland of the bird. Single doses of 100 µg of testosterone were then injected into the pectoral musculature. Animals were killed one hour after treatment and samples taken for assessment of AR levels.

Multiple dose study: Groups of nine birds were assigned to the following treatment regimens:

Group	Testosterone	Test material
1	No	No
2	Yes	No
3	Yes	Injected
4	Yes	Topical application

Testosterone was administered by implantation of a propionate-filled silastic capsule. The appropriate dose of test material was given once daily for 21 days either by subdermal injection close to the uropygial gland of the bird or by topical application to the skin covering the gland. Animals were killed after completion of the treatment period and samples taken for assessment of AR, C12-diol and blood testosterone levels.

Endpoints/data interpretation

AR level: measured by an exchange technique using either [³H]R1881 or [³H]mibolerone. Both inactivated and activated receptors were measured. In the case of the single dose study, the “activated to inactivated” ratio was calculated.

C12-diol level: measured by a capillary column gas liquid chromatography; expressed as a percentage of total diols in the secretory product.

Clear evidence of the activation of AR in the uropygial gland by testosterone administration and its dose-dependent inhibition by the test material was shown in the single dose study. In the longer-term study, effects of testosterone on AR and C12-diol were as expected in the positive control, whilst topical administration of test material again exerted a local, but not systemic, inhibitory action.

RODENT SUBMANDIBULAR GLAND SENSITIVITY

Introduction

Katsukawa *et al.* (1989) performed a number of experiments to investigate the effects of androgen exposure on the development of the male and female murine submandibular gland. The activities of androgenic metabolising enzymes (5α-reductase, α-hydroxysteroid dehydrogenase (3α-HSDase)) and the

nuclear binding of 5 α -dihydrotestosterone (DHT) were measured in the gland following pre-treatment with different androgens.

This model was not intended as a screening system for the identification of environmental chemicals. It only investigated the effects of recognised androgenic hormones. Nonetheless, endpoints of potential usefulness for assessment of androgenic activity were derived and the procedure has therefore been briefly reviewed.

Method

ICR mice between two and four weeks of age were used. They were maintained under standard housing conditions.

Experiment 1:

The androgenic response of the submandibular gland was examined by treating male and female mice with testosterone (T) for five-day periods from days 9, 16 or 23 of age. Esteropeptidase activity was measured approximately 24 hours after the last injection.

Experiment 2:

In order to study the responsiveness of esteropeptidases, animals were assigned to the groups detailed below from day 13 of age. Enzyme activity was measured approximately 24 hours after the last dose.

Experiment 3:

The effects of long-term androgen treatment on responsiveness of 5 α -reductase and 3 α -HSDase was investigated using male mice treated in accordance with the schedule used for Experiment 2.

Experiment 4:

Effects of long-term androgen treatment on nuclear androgen binding was examined using male and female mice treated with T or sesame oil from days 18 to 27 of age. Receptor assays were performed on day 28 of age. In addition, age-related changes in binding were investigated in normal animals of two to four weeks of age. All animals were treated with T two hours before assay.

Group	Animals	Initial 10-day pre-treatment	Subsequent testosterone treatment for 5 days	
1	Normal males	None	Yes	No
2	Castrated males	T	Yes	No
3	Castrated males	DHT	Yes	No
4	Castrated males	5 α -androstane-3 α , 17 β -diol	Yes	No
5	Castrated males	Sesame oil	Yes	No
6	Normal females	None	Yes	No
7	Ovariectomised females	5 α -androstane-3 α , 17 β -diol	Yes	No
8	Ovariectomised females	Sesame oil	Yes	No

Tissue preparation: submandibular glands were homogenised, centrifuged, and supernatant taken for enzyme assays. A similar process was used to obtain a tissue pellet for nuclear androgen-binding assay.

Results were analysed using Student's t-test.

Endpoints/data interpretation

Esteropeptidase activity: measured using N-benzoyl-DL-argine-p-nitranilide as substrate.

5 α -reductase and α -hydroxysteroid dehydrogenase activities: measured using [¹⁴C]-labelled testosterone and tritiated 5 α -dihydro-1,2,4,5,6,7-testosterone. Activity was expressed in terms of nanograms of relevant compound produced from the substrate.

Nuclear binding of 5 α -dihydrotestosterone: measured using tritiated DHT and counting in a scintillation counter. Protein and DNA binding were determined.

Development of androgen sensitivity occurred in mice after the first two weeks of life, with intact males showing a more marked effect than females or castrated males. Pre-treatment with androgens in Experiment 2 similarly enhanced the subsequent responsiveness of the gland. The authors suggested that this indicates that a continuous exposure to androgens accelerated the development of responsiveness of the gland, at least early in life. Nuclear androgen binding was found to be low until four weeks of age, when a marked increase was seen, particularly in males. This was thought to possibly explain the differences in sensitivity noted during androgen administration.

RODENT MATURE/IMMATURE MALE RESPONSES

Introduction

Cook *et al.* (1993) reported on a series of studies designed to elucidate the mechanisms of Leydig cell tumourigenesis by linuron in rats. Flutamide was used as a positive control. The aspect of the investigation reported here related to the comparison of effects in immature and adult male animals. Rats were treated for two weeks with either linuron or flutamide, followed by measurement of accessory sex organs and serum hormonal levels.

Method

Male CD rats were allocated to 10 groups so that the initial mean body weights were similar. Immature and mature individuals were dosed from days 32 to 45 and days 93 to 107 of age, respectively. Flutamide and an appropriate vehicle control was administered by subcutaneous injection. For the linuron work, treated animals and control and pair-fed control animals were dosed by oral gavage. Animals were killed the morning after the last dose and blood collected. The weights of prostate, seminal vesicles and coagulating glands were recorded. Serum was obtained by centrifugation and stored deep-frozen until analysis for testosterone, oestradiol and lutenising hormone concentration using radioimmunoassay procedures.

Endpoints/data interpretation

Organ weights: expressed as bodyweight-relative values.

Serum hormone levels: by radioimmunoassay.

Cook *et al.* reported decreased bodyweight-relative values for epididymides, prostate, seminal vesicle and coagulating gland in immature flutamide-treated animals. Increases in all levels of all serum hormones examined were also observed. In mature animals treated with flutamide, a similar picture was seen, except that oestradiol concentrations were not affected.

For the linuron-treated immature animals, decreased bodyweight-relative organ weights were seen when compared with their pair-fed controls; a reduction was also observed for all except the epididymides when

compared with the normal controls. Serum hormonal levels were found to be unaffected by treatment with linuron in these immature animals. When the sexually mature males were considered, effects on organ weights were again apparent, whilst reductions in oestradiol and lutenising hormone concentrations were seen.

Age significantly affected the response to treatment with either chemical, although anti-androgenic activity was clearly detectable in each case. The authors commented that detection of anti-androgenic activity can therefore be optimised by ensuring that mature and immature animals are used during the screen of chemicals.

MOUSE SEMINAL VESICLES

Introduction

Broulik and Horky (1988) studied the effects of minoxidil, an antihypertensive vasodilator, on the seminal vesicles of mice. This drug was elicits hirsutism and abnormalities of menstruation in some women following long-term treatment. The use of the animal model was justified on the basis of a high sensitivity to androgens by mouse seminal vesicles.

Method

Castrated mice of the H strain were maintained under standard laboratory conditions. Animals were assigned to seven groups, each of eight animals, and given the following treatment regimen:

Group	Castrated (days before treatment)	Treatment
1	Not castrated	None
2	21	None
3	21	Testosterone isobutyrate - weekly by subcutaneous injection
4	21	Dihydrotestosterone - every other day by subcutaneous injection
5	21	Minoxidil - low dose - constantly in the diet
6	21	Minoxidil - high dose - constantly in the diet
7	1	Minoxidil - high dose - constantly in the diet

Animals were weighed at the start and completion of treatment. After 21 days' treatment, animals were killed and the adrenals and seminal vesicles weighed. Seminal vesicles were re-weighed after drying to a constant mass (dry weight). Data were analysed by Duncan's test.

Endpoints/data interpretation

Organ weight: expressed as absolute and bodyweight-relative values.

Seminal vesicle dry weight: weighed after oven drying.

In undosed castrated animals, a marked decrease in seminal vesicle weight was seen compared to the control values, when expressed in either absolute or bodyweight-relative terms. Administration of dihydrotestosterone returned the weights to similar to control values, whilst testosterone caused an increase above control values. In contrast, the seminal weights of minoxidil-treated animals were similar to the untreated castrated animals, indicating a lack of androgenic activity for this chemical. Seminal vesicle dry weight reflected the findings for the wet tissue. Finally, adrenal weights were shown to be unaffected by any of the treatments

HAMSTER FLANK ORGAN

Introduction

Brooks *et al.* (1991) reported on a series of experiments in which the hamster flank organ was used to assess the anti-androgenicity of a new 4-azasteroid drug (methyl 3-oxo-4-methyl-4-aza-5 α -androst-1-ene-17 β -carboxylate) and 17 α -propyltestosterone. The authors quoted previous papers relating to the structure of the flank organ and the need for continued androgenic stimulation in order to maintain its integrity as the basis for this test method.

Method

Male Golden Syrian hamsters were obtained from commercial sources and housed under standard conditions in groups of four to seven. On the day before commencement of treatment, animals were castrated and the area of the left flank organ shaved with clippers. Control animals received daily subcutaneous injections of the vehicle, cotton seed oil. Other groups received a similar injection containing either testosterone propionate or dihydrotestosterone propionate, at dosages designed to give an approximately equal degree of stimulation to the flank organs. In addition, the appropriate test material was applied topically to the left flank organ at a range of dosages: the right flank organ remained untreated. Animals were humanely killed one day after completion of the 21-day treatment period. The weight of the prostate gland was recorded, and the length and width of the darkly pigmented oval spot outlining each flank organ was measured using callipers. Skin samples, including the flank organs, were taken into formalin. In some of the experiments these samples were processed histologically, and sections taken through the centre of the gland and perpendicular to the surface were stained using haematoxylin and eosin. The total surface area of the sebaceous glands on each section was calculated using microscopic image analysis techniques.

Data were analysed by one-way analysis of variance to obtain a pooled measure of experimental variability. Trend was assessed using regression slopes based upon arithmetic, arithmetic-logarithmic and ordinal scalings. Probability values were derived using the Tukey-Ciminera-Heyse stepwise trend test.

Subsequent development

Cabeza *et al.* (1995) investigated the androgenic activity of a synthetic progestin, levonorgestrel, and its 5 α -reduced derivative on the hamster flank organ. Groups of four animals were used. Male hamsters were castrated seven days before commencement of treatment. The flank organs of each animal were shaved before application. Animals received either vehicle alone, testosterone (positive control), levonorgestrel, or testosterone in combination with either test material. A group of intact animals was also included as a reference, untreated control. Treatments were applied by pipette to the right organ daily for 15 days. The left flank organ received daily administration of the solvent alone. On completion of the treatment period, the organ diameter and weight were recorded at necropsy.

The excised glands were incubated in Erlenmeyer flasks (four whole glands from the same treatment in each) with U[¹⁴C] glucose and non-radiolabelled glucose for two hours. Experiments were performed in duplicate. Lipids were extracted by placing the glands in Folch's solution for 24 hours and adding water to the organic phase of the incubation medium after removal of the glands. The aqueous layer was then removed and heated to dryness. Extracted lipids were weighed and radioactivity determined by liquid scintillation count.

Data were analysed by one-way analysis of variance.

Endpoints/data interpretation

Brooks *et al.* (1991):

Flank organ area: calculated from length (L) and width (W) measurements of the flank organ at necropsy using the formula:

$$\text{Area} = \pi(L/2 \times W/2)$$

Sebaceous gland area: calculated by image analyser from the outline of the sebaceous gland area on microscopic sections.

Treatment with either test material resulted in a significant decrease in the area of the left flank organ at all dosages examined, when compared with the controls. Histopathological assessment of sebaceous gland area supported this finding. Neither prostate weight or right flank organ size was affected by the test material applied to the left flank organ.

Cabeza *et al.* (1995):

Flank organ diameter: by measurement at necropsy.

Flank organ weight: by measurement at necropsy.

Radiolabelled glucose in extract: expressed as pM/gland/hour of incubation.

Lipid content in extract: expressed as mg/gland.

Testosterone acted to restore the appearance of organs from castrated animals. Both test materials increased the diameters of the spot, although co-administration with testosterone did not appear to cause an additive response. Data for organ weight changes were not presented; however, the lipid content and U[¹⁴C] glucose incorporation were generally found to reflect the changes seen in spot diameter. There was some evidence to suggest a minimal, but not statistically significant, change in the size of the contralateral organ of castrated animals following treatment, suggesting a limited degree of systemic absorption during the course of the treatment period.

DOG PROSTATE ULTRASONOGRAPHY MODEL

Introduction

Cartee *et al.* (1990) reported on an experiment to evaluate the value of transabdominal ultrasonography in monitoring prostatic involution in the dog following treatment with an anti-androgen.

Method

Twenty-five adult male beagle dogs were maintained under standard housing conditions. Animals were allocated to groups receiving varying dosages of either flutamide or hydroxyflutamide, in gelatine capsules or empty capsules alone (control), by daily oral dosing for 47 days. The prostate glands of all dogs were scanned transabdominally using 10 MHz and/or 5 MHz ultrasound transducers. Probe orientation was adjusted to provide the maximum transverse cross-sectional profile. This image was then electronically captured and the image and dimensions recorded on videotape or radiographic film. Rectal palpation and manipulation were performed during scanning in order to confirm the margins of the prostate gland. Examinations were performed, without reference to treatment group, on seven occasions in the two weeks before treatment, twice weekly during the treatment phase, and immediately before necropsy of the dogs. On the first day of scanning and immediately before necropsy, the right testis was also scanned and measured. After dissection, the dorso-ventral and transverse physical dimensions of the prostate were measured using callipers. The gland was then placed in physiological saline in a water bath and again scanned ultrasonically. Tissue was taken for histological examination.

In order to calculate surface areas of scanned images, the glands were assumed to be either spherical, oblate or prolate ellipsoids and a largest and smallest dimension approach was used for calculations. The resulting cross-sectional area estimate (CSAE) data were statistically analysed using one-way analysis of variance to compare the last four scans during the treatment period, the pre-humane-killing scan and the water-bath immersion scan; Fisher's method of least significant differences was applied to between-treatment differences. Testicular size measurements were compared using the Newman-Keuls multiple-range test.

Endpoints/data interpretation

Prostate CSAE: calculated from the largest and smallest diameter measurements by ultrasound or physical examination.

Testicular size: calculated from measurements by ultrasound or physical examination.

Prostate histological appearance: by microscopic examination.

Treatment elicited a dosage-related change in the testis size and in prostate involution at the highest dosage of either drug. Comparison of the CSAE from the last four in-life scans, the water bath scan and the physical measurement at necropsy showed that ultrasound scans give a smaller value than manual measurement, but the difference did not attain statistical significance.

Histopathological examination showed less well developed prostatic acini and reduced epithelial height in all drug-treatment groups.

Although noting day-to-day variability in the ultrasonic measurements in the dog prostate, the authors considered the use of ultrasonography to be a highly effective method of monitoring prostate size and of monitoring involution over time.

DOG SIALIC ACID DEPLETION TEST

Introduction

Jain and Dixit (1986) investigated the changes in sialic acid level in the castrated and intact dog following administration of a plant extract possessing hormonal activity derived from the dried leaves of *Aloe barbedensis* (Mill) using ethanol extraction. The authors considered this of interest given the reported involvement of sialic acid with sperm capacitation and the apparent links between regulation of its epididymal plasma concentration and testosterone administration.

Method

Thirty-six adult male dogs were assigned to six groups, each of six animals, and given the following treatment protocol for 60 days:

Group	Plant extract treatment	Testosterone propionate	Castrated (Yes/No)
1	-	-	N
2	2 g of 50% extract/day	-	N
3	2 g of 50% extract/day	2 mg/day twice a week for 8weeks	N
4	-	-	Y
5	-	2 mg/day twice a week for 8weeks	Y
6	2 g of 50% extract/day	2 mg/day twice a week for 8weeks	Y

Twenty-four hours after completion of treatment, animals were killed and the testes (where appropriate), epididymides and prostate removed. Sialic acid content was analysed, using methodology not reported in this paper, for the testes, caput-, corpus- and cauda-epididymides and prostate glands.

Endpoints/data interpretation

Sialic acid concentration: by chemical analysis.

Administration of the plant extract to intact dogs significantly reduced the sialic acid level in each of the tissues analysed. Co-administration of testosterone resulted in an increase in the level, although it remained below that of the controls. Castration was resulted in a reduction in the concentrations of sialic acid, whilst the anti-androgenic activity of the plant extract was clearly apparent when testosterone was co-administered with the plant extract to castrated animals.

IN VIVO SYSTEMS RELEVANT TO ASSESSMENT OF TOXICITY TO HUMANS

NON-SPECIFIC

HUMAN CELL LINE SCREENS

Introduction

Model systems, based upon human cell lines, that are specifically designed to detect agonist or antagonist interactions with either the oestrogen or androgen receptor are presented in the relevant sections below. In addition, a small number of test systems or models using human cell lines focusing upon other endpoints or mechanisms of action have been reviewed. Information relating to these are presented in the following section.

T-47D cell lines

Introduction

Markiewicz and Gurpide (1994), in a paper investigating progestagenic activities of steroidal drugs, described the specific stimulation of alkaline phosphatase in T47-D cells as a marker for progestagenic activity. The method used was a microtitre plate assay. Both natural and synthetic chemicals were used, including progesterone, diethylstilboestrol and a number of 19-norsynthetic progestins together with a synthetic antiprogesterin, RU486.

Method

Cells were maintained in DMEM (Dulbecco's Modified Eagles Medium) [ed?], supplemented with streptomycin, penicillin and fetal bovine serum (FBS). Twenty-four hours before use, the medium was replaced with DMEM containing FBS (stripped using dextran-coated charcoal) to remove endogenous steroids. At the start of the exposure period, cells were harvested with trypsin and plated out on a 96-well, flat bottomed tissue culture plate. Test compound solutions were added to the wells, as appropriate, after which plates were incubated at 37°C in a humidified, CO₂-rich atmosphere for 72 hours. Medium was then removed and cells washed twice in PBS-sodium phosphate. After shaking out, cells were fractured by freezing to -80°C. Following thawing, plates were assayed for alkaline phosphatase activity using *p*-nitrophenolphosphate with the reaction being monitored over a 100-minute period. Semilogarithmic plots of optical density vs. concentration were constructed. Maximal effect, slope factors and the EC₅₀ values were then calculated by computer.

Subsequent development

White *et al.* (1994a) developed an assay system for progestagenic and androgenic chemicals using the T47-D cell line by stably transfecting a DNA cassette containing a synthetic steroid-inducible promoter into an Epstein-Barr virus (EBV) episomal vector. The promoter controlled the expression of a bacterial chloramphenicol acetyltransferase (CAT) gene (GRE5-CAT).

T47-D cells were propagated in RPMI 1640 media supplemented with fetal bovine serum. At approximately 50% confluence, cells were transfected by calcium phosphate co-precipitation, then passaged with hydromycin being added for selection 48 hours later. CAT-positive, antibiotic-resistant cultures were propagated further.

Cells were plated and exposed to test chemical-containing medium which was refreshed every 24 hours. Cells were harvested after 40-48 hours and CAT activity determined either by thin layer chromatography separation with chemical elucidation or *in situ* determination by lysis and subsequent colourimetric

determination using butyl CoA, chloramphenicol and DTNB. Plates were read before and after incubation for not less than 180 minutes.

Endpoints/data interpretation

Markiewicz and Gurpide (1994):

Alkaline phosphatase activity: determined by *p*-nitrophenolphosphate reaction; maximal effect, slope factors and EC₅₀ values were calculated.

In general, the assay in conjunction with computer-assisted analysis could differentiate between the activities of the compounds tested. As expected, some of the steroids behaved as full agonists whilst others showed only partial activity. Efficacy EC₅₀ values and slope factors were used to characterise the agonistic effects.

White *et al.* (1994a):

CAT activity: Determined colourimetrically or by thin layer chromatography.

Virtually no CAT activity was detected by either method in transfected cells in the absence of a hormone. Dexamethazone poorly induced activity, but strong responses were given by 100 nM progesterone and 100 nM dihydrotestosterone treatment. Following exposure to a range of progestagens at concentrations from 0.1 to 100 nM, a maximal stimulation (3.5-fold) was observed for 100 nM progesterone. The agonist, RU27987, was approximately twice as effective as progesterone. T47-D-GRE5 cells responded to 1 µM dihydrotestosterone under the same conditions with a maximal stimulation of only 1.5 fold after three hours. In terms of sensitivity, the low background levels using the chromatographic method ensured its superiority to the colourimetric method.

ZR-75-1 Human breast cancer cell line

Introduction

Poulin *et al.* (1990) investigated the multiple, steroid receptor mediated activities of a series of synthetic “progestins” (medroxyprogesterone acetate, megestrol acetate, norethindrone, norgestrel, chlormadinone acetate and cyproterone acetate) on breast cancer growth using the human ZR-75-1 cell line. This line possesses functional oestrogen (ER), androgen (AR), glucocorticoid (GR) and progesterone (PgR) receptors. The objective of the study was to assess and quantify relative contributions of different steroid receptor systems to the control of cell proliferation by the progestins.

Method

ZR-75-1 cell cultures in late exponential phase were harvested and re-suspended in medium supplemented with bovine calf serum (stripped using dextran-coated charcoal) and, where required, bovine insulin. Cells were plated and allowed to adhere for 48 hours before steroids and/or antagonists were added. Cells were incubated for 10 to 15 days, with medium changes every second day, before cultures were trypsinised and cell numbers determined by Coulter counter. The specific uptake of [³H]E₂, [³H]R1881 and [³H]R5020 in the presence of various competitors was also assessed to determine relative binding for oestrogen, androgen and progestin specific sites. Cells were grown to near confluence in media supplemented with E₂ for the determination of progesterone binding sites. Then they were incubated, in triplicate, for 60 minutes with the appropriate radiolabelled chemical and a range of increasing concentrations of competitors.

Endpoints/data interpretation

Cell proliferation: determined by Coulter counter.

Relative binding affinity: measured by competitive binding assay of radio ligand binding with Scatchard analysis.

Addition of insulin completely reversed the inhibition due to the interaction of R5020 with PgR in the ZR-75-1 cells. The antiproliferative effect of R5020 only occurred under E₂-stimulated conditions. These characteristics permitted the study of the relative contribution of PgR in the effect of the progestins, investigated by evaluating the effect of insulin and/or oestrogen addition on growth response at the end of a 15-day incubation period. The six “progestins” were recorded as having very different patterns of effect on the proliferation of the cells.

ANIMAL TISSUES AND PRIMARY CELL SCREENS

Introduction

A large number of *in vitro* systems have been developed that use tissues or primary cell cultures from various non-human species to study the effects of chemicals. These models can show the potential for interference with developmental or metabolic functions at the cellular level, thereby suggesting the possibility that a chemical may possess some form of sex hormone-disruptive activity. In some cases the models do not give precise information as to the mechanisms of action, nor do they address the complex interactions that can occur within an *in vivo* system. As such, these tests may have a role in the screening of chemicals but are unlikely to address all the aspects necessary to characterise the potential hazard of a chemical.

Bovine uterine culture

Tiemann and Tuchscherer (1995) reported on the use of a bovine uterine culture system to detect effects of DDT, methoxychlor and hexachlorocyclohexane.

Method

Uterine stromal, muscle and epithelial cells were cultivated to approximately 50% confluency. The medium was then changed to one with or without test chemical at a range of concentrations. After 24 hours' exposure, tritiated thymidine was added. The cells were harvested 12 hours later and incorporation of radiolabel was assessed.

Endpoints/data interpretation

DNA synthesis: measured by tritiated thymidine incorporation.

There was a dose-dependent decrease in incorporation in response to exposure to the test chemicals, with differences being seen for each type of cell. Epithelial cells showed a significant increase in label incorporation for DDT at 1 ng (37%), 10 ng (26%) or 100 ng (35%) per 100 µL whilst hexachlorocyclohexane caused increases at 1 or 10 ng/100 µL (25%). Methoxychlor showed 144% stimulation at 1 or 10 ng/100 µL.

Monkey ovarian model

Introduction

Bengtsson and Mattison (1989) reported on the use of an *in vitro* monkey ovarian cell culture system to study the metabolism of 7,12-dimethylbenz(*a*)anthracene (DMBA).

Method

Two sexually mature rhesus monkeys were given unilateral intraovarian injection with pregnant mares' serum gonadotropin (PMSG), under anaesthesia, 48 hours before humane killing and removal of ovaries. Following collagenase treatment, cells from each ovary were checked for viability, filtered using a 100 µm nylon filter, and placed on top of a discontinuous Percoll gradient. After centrifugation to separate the cells into separate bands, each band was assayed for DMBA-monoxygenase activity by culturing for 16 hours followed by addition of tritiated DMBA for seven hours and assessment of activity by radiomeasurements.

Endpoints/data interpretation

DMBA-monoxygenase activity: expressed as pM/hour × 10⁶ cells.

Increased DMBA activity was seen only in cells taken from the ovary stimulated by PMSG before humane killing. Similar activity has been reported in humans, but there was an indication that the metabolically competent cells may be different between the species.

Rodent ovarian models

Introduction

Mattison (1993) reviewed the potential sites of reproductive sensitivity in the female and the implications of such effects on the risk assessment process for causative agents. This paper included consideration of the different parts of the ovary that might be influenced by xenobiotic interactions. Cell types thus identified included oocytes, granulosa, thecal and stromal cells. The study of such chemical interactions may require both *in vivo* and *in vitro* techniques. A number of rodent model systems that could be used are reviewed below.

Rat models

Method

Gangnerau *et al.* (1991) developed a culture system to explore the changing metabolic and functional capacity of ovaries to respond to chemical stimulation during the fetal and early neonatal periods.

Pregnant Wistar rats were laparotomised under anaesthesia and fetuses delivered aseptically. Fetal gonads were immediately removed and either immersed in culture medium or frozen in liquid nitrogen until assay for binding studies. Similar procedures were performed to obtain neonatal gonadal tissue. Where appropriate, gonads were cultured in a modified M199 medium which was replaced every 24 hours.

The responsiveness of ovarian tissue to LH was evaluated using tissue cultured over 24 hours, taken from day 18, 20 and 22 fetuses and from day 2, 4, 6 and 8 neonates. The effects of addition of LH and 3-isobutyl-1-methyl-xanthine (IBMX), either alone or in combination, to the culture medium was investigated by assay for oestrogen production. The ability to produce progesterone was similarly assessed by culturing tissues from day 18, 20 and 22 fetuses or day 2, 4 and 6 neonates with LH or IBMX for 48 hours. In these studies all media were supplemented with spironolactone to inhibit further metabolism of the progesterone into oestrogens.

Other experiments conducted included monitoring the effects on 3 β -HSD and oestradiol levels of culturing tissues from fetuses at days 18 and 22 and neonates of 2, 4, 5 and 9 days of age in media containing (Bu)₂cAMP or LH. The effects of a number of other chemicals on ovarian tissue function were also investigated using this culture system; these included forskolin, cholera toxin and vasoactive intestinal peptide (VIP), which were tested on 18-day-old fetal ovaries.

The changing ability of ovarian tissue to bind hCG was also monitored for days 18 and 20 of gestation and days 7 and 10 after birth. The effects of incubation with (Bu)₂cAMP were also investigated for tissues from day 18 of gestation fetuses. Finally, the neuramidase activities of fetal and neonatal tissue were measured, with and without incubation, in medium containing (Bu)₂cAMP.

Radioimmunoassays were performed, using standard procedures, for testosterone, oestradiol or oestrone levels. In addition, 3 β -hydroxysteroid dehydrogenase/ isomerase (3 β -HSD) activity was determined by the conversion of [³H]pregnenolone to [³H]progesterone. This involved homogenisation of the gonads, addition of known quantities of labelled and unlabelled pregnenolone to the homogenate and incubation for 10 minutes. Steroids were extracted using ethyl acetate and separated by silica gel-TLC. Visualisation was by UV-light for progesterone and iodine vapour for pregnenolone. Metabolites were extracted and quantified by scintillation counter. Similarly, homogenised gonadal tissue was assayed for hCG binding using a competitive binding assay with [¹²⁵I]-labelled hCG. Neuramidase activity was assessed by incubation of tissue homogenate or respective cellular fraction in sodium acetate buffer with bovine sialyllactose. After two hours' incubation at 37°C, the mixture was cooled in an ice bath and assessed for sialic acid level by the method of Warren.

Data were analysed by Student's t-test and one-way ANOVA (Scheffé F-test).

Subsequent development

Bengtsson *et al.* (1992) reported on a methodology that permitted the separation and culture of different cell types from rat ovaries. The authors intended to use this model to assist in the investigation of the cell types responsible for the bioactivation of the mutagenic, carcinogenic and teratogenic chemical dimethylbez(a)anthracene (DMBA) and the hormonal regulation of metabolism.

Animals used were Sprague-Dawley rats housed under standard conditions; both mature and immature animals were studied. Mature animals were killed four days after receipt of a single subcutaneous injection of pregnant mare's serum gonadotropin. Immature animals were similarly dosed at 26 days of age and killed on day 29. Appropriate vehicle control animals were also used. Ovaries were collected into buffered saline, treated with collagenase, and separated on a 20-60% discontinuous gradient medium. Centrifugation at 400 g for 20 minutes was used to distribute cells through the gradient medium into four or five bands. Cells were taken from each band and transferred onto plate cultures at 37°C under air, at 95% humidity. Samples were also taken, fixed, and ultra-thin sections prepared using uranyl acetate and lead citrate.

Cells were cultured in the presence or absence of hCG or testosterone, alone or in combination. Quadruplicate samples were used in all cases. After 24- or 48-hours, [³H]DMBA was added for 12 hours to the cultures and its metabolism subsequently assessed by radioassay. Progesterone and oestradiol levels were also analysed in the culture medium using radioimmunoassays.

Endpoints/data interpretation

Gangnerau *et al.* (1991):

Testosterone, oestrone or oestradiol tissue levels: by radioimmunoassay.

3 β -HSD activity: determined by the conversion of [3 H]pregnenolone to [3 H]progesterone.

hCG binding: measured by competitive binding with [125 I]-labelled hCG.

Neuramidase activity: by sialyllactose conversion.

Investigation of the *in vitro* responsiveness to LH or IBMX stimulation of ovarian tissues, taken from animals of different ages, identified that the capacity to produce measurable levels of oestrogens was not achieved until between days 4 and 6 post partum. Progesterone production could be induced from day 4 post partum. Addition of (Bu) $_2$ cAMP to the culture medium enhanced 3 β -HSD activity three- to five-fold on day 18 of pregnancy and day 9 post partum respectively; oestradiol production was also seen at these times. LH had no effect upon 3 β -HSD activity before day 5 post partum. Both forskolin and cholera toxin induced progesterone production, particularly in the presence of IBMX and spiro lactone whilst VIP significantly elevated 3 β -HSD activity and, at the highest dosage, resulted in oestradiol production. Ovarian binding of hCG was only observed for days 7 and 10 after birth in untreated tissue. However, when fetal tissue was pre-treated for 48 hours with (Bu) $_2$ cAMP, specific bonding became apparent. In contrast, no evidence of any changes in neuramidase activity were detected between fetal and neonatal tissue.

The apparent developmental differences in ovarian metabolic capability for oestrogen and progesterone may actually arise from the relative sensitivities in the respective radioimmunoassays used, which would explain differences between their *in vitro* work and other *in vivo* data. Overall, it was suggested that all steroidogenic enzymes are probably present in the ovary from day 4 post partum and that the lack of earlier responsiveness may arise from the absence of receptor expression.

Bengtsson *et al.* (1992):

DMBA mono-oxygenase activity: assessed by radioassay of [3 H]DMBA metabolism.

Progesterone and oestradiol levels: measured by radioimmunoassay.

Histopathology: using ultra-thin sections.

On the basis of distribution of cells through the discontinuous density gradient, pre-treatment of mature rats with PMSG did not appear to influence the selective proliferation of any particular cell type. In the case of the immature animals, two cell layers (at the 30-40% and 40-50% interface) were enriched. Measurements of DMBA mono-oxygenase activity showed this not to be affected by hCG in untreated mature rats; However, testosterone caused a marked decrease in activity in cells of the high-density fraction whilst an inactive fraction from the 20-30% interface showed a marked increase in activity levels. In PMSG-treated mature animals, activity levels of the 40-50% fraction cells decreased in the presence of hCG or testosterone whilst the less active fractions increased on exposure to these agents. In the immature animals, pre-treatment with PMSG showed high levels of DMBA mono-oxygenase activity. This was decreased by hCG and totally extinguished by testosterone. Hormonal assays showed PMSG to enhance levels of progesterone and oestradiol significantly whilst testosterone, but not hCG, induced levels of oestrogen for mature rat ovaries. Progesterone content was not influenced to the same extent.

Light and electron microscopy of the cell layers, and comparison with intact tissues, suggested that there was overlap of cell types but also demonstrated differences between controls and pre-treated populations. The authors commented that although the separation of cell types was not complete by this method, the use of a density gradient permitted enrichment of certain cell populations at the different interfaces.

Mouse model

Torrance *et al.* (1989) developed a technique for the isolation and culture of intact mouse pre-antral ovarian follicles. The follicle is the basic functional unit of the mammalian ovary, being responsible for oocyte growth and maturation and the production of sex steroids, and the technique used mimicked the physiological conditions for the intact follicle in which the normal three-dimensional relationships were maintained between the different cell types.

This paper related only to the culture technique and, as such, included no assessment of the responsiveness of the cultured cells to exogenous chemicals. Nor did it provide other than histopathological endpoints.

Method

Animals used were aged eight to 11 days and were obtained from crossing CBC/Ca males with C57BL/6 females. Animals were killed by decapitation and the ovaries removed and placed in a watch-glass containing cold media. Subsequent stages were performed using sterile techniques in a laminar flow hood. The ovaries were dissected and incubated with gentle rotation at 37°C for 30 minutes, then centrifuged and the supernatant removed. Suspension and centrifugation were repeated. Subsequently, follicles were isolated by repeat pipetting under a dissecting microscope. Freed follicles were harvested frequently and filtered through a nylon mesh. Follicles were pelleted by gentle centrifugation and then re-suspended in fresh medium. The pellet was broken up by gentle rotation in a watch-glass and 20 µm samples pipetted into the wells of a plate. The process was repeated until 16 samples had been obtained. After removal of excess fluid from the wells, collagen gel was added. This gel was prepared from the tail tendons of rats by mixing tendon samples with acetic acid at 4°C for 48 hours and centrifuging for one hour at 2000 g. The resulting solution could be stored for up to eight weeks before use. Immediately before addition to the follicles, this solution was mixed with serum and a Hepes-buffered medium and the pH adjusted to a normal physiological value. After addition of the gel, the culture was incubated for two to three minutes to permit gel setting. The tissue-containing follicles were then transferred to further well-plates and additional gel added. This double gelling was found to be necessary to avoid follicle losses resulting from collagen gel contraction during processing. Gels were then transferred to a Linbro tissue culture plate containing culture medium and incubated at 37°C with 5% CO₂ in a humidified incubator. After 24 hours, and then at three-day intervals, the medium was changed. Follicles were monitored using inverted phase-contrast microscopy.

At termination of the culture period, gels were fixed overnight in Bouin's fluid, embedded in paraffin wax. Haematoxylin- and eosin-stained sections were examined by light microscopy and classified on the basis of the number of cell layers surrounding the oocyte. Note was also made of the number of intact, damaged or disrupted follicles.

Endpoints/data interpretation

Follicle development/integrity: assessed by light microscopy.

The number of follicles in the cultures declined with time and associated with the gradual reduction, from the fifth to the eighth day, of unhealthy follicles. By day 14 of culture, however, the number of healthy follicles was showing a marked decrease. A marked shift in the type of follicles was seen during the course of the 14-day culture period, with the smaller stages gradually disappearing and larger ones emerging, suggesting that the test conditions permitted normal development of the follicles over this period. The problems relating to maintenance of late stage follicles may arise from the medium used, which contains little in the way of gonadotrophins or sex hormones and may therefore be unable to support these stages of development.

ANIMAL CELL LINE SCREEN

Introduction

Only one established cell line screen was reviewed that did not have endpoints directly related to oestrogenic or androgenic activities. This was a recombinant receptor/reporter gene assay used by Zacharewski *et al.* (1995) to assess the Ah-receptor-mediated, dioxin-like activity of two pulp and paper mill effluents, black liquor (a recovered by-product from the pulping industry) and a methanol-extracted effluent fraction (MF).

The established cell line, Hepa1c1c7, was used. This is a mouse hepatoma-derived line which endogenously features the Ah-receptor. In this assay the cells were transfected with a luciferase dioxin-responsive element (DRE) regulated-reporter with β -galactosidase as an internal control for transfection efficiency.

Method

Cell lines were maintained in DMEM in a 5% CO₂ humidified environment at 37°C.

Cells were plated on dishes at approximately 50% confluency before transfection in media supplemented with 10% fetal bovine serum (stripped using dextran-coated charcoal). After six hours, cells were transiently transfected with pCH110 (β -galactosidase expression vector), pGudLuc1.1 (luciferase reporter) and pBS as carrier DNA using a calcium phosphate co-precipitation technique. After 20 hours, the plates were washed and the media replaced.

The AhR-mediated activity of black liquor and MF were studied by their effect on luciferase activity relative to a concentration of TCDD. Cells were treated with test material and harvested after 24 hours for luciferase and β -galactosidase activity determination. Treatments were performed in duplicate, with two samples being taken from each dish and the mean used for calculation of percentage relative to the maximum induction reported with 1 nM TCDD. Concentrations for test mixtures were deduced by comparison of the dose-response curves and reported in TCDD equivalents (defined as the amount or concentration of TCDD within a sample necessary to elicit a half-maximal response).

Endpoints/data interpretation

β -Galactosidase: used as an internal standard for differences in transfection efficiency.

Luciferase: expressed as a percentage of activity attained with 1 nM of TCDD.

The dioxin-like potential of both the black liquor and MF was shown by AhR bioassay, and it was mediated solely through the Ah-receptor. A dose-dependent induction of pGudLuc1.1 mediated activity was seen, with a detection limit of approximately 80 pg TCDD per plate. This was reported as comparable to or in excess of the sensitivity of other assays.

Co-treatment with TCDD resulted in no net difference from TCDD alone, indicating absence of inhibitory effect. To show receptor-specific mediation of luciferase activity, two mutant strains of Hepa 1c1c7 were transfected. These featured mutations of the structural genes encoding for the receptor and its nuclear translocator protein. The net result of these mutations was that there was no net increase in luciferase activity.

OESTROGEN-RELATED

HUMAN PRIMARY CELL SCREENS

Introduction

Clearly the best model for assessing the toxicological significance of sex hormone-disrupting chemicals to a given species is to study effects in individuals of that species. In the case of non-medicinal chemicals, this is generally not appropriate in humans. It is, however, possible to use a number of cell types of human origin to investigate responses in the *in vitro* situation (see Section 0). These models have potential limitations due to their reliance on immortalised cell lines; however, the use of human primary cells might overcome some of the reservations. Holinka (1988) presented a method for establishing both primary and secondary endometrial cell cultures and reviewed the potential uses of such cell lines in investigating the effects of chemicals on human cells and the study of hormonally regulated gene expression during cell differentiation. Human primary endometrial cells have since been used to investigate the activities of oestrogenic chemicals. The uterine models reviewed here are those of Casslén and Harper (1991) and Markiewicz *et al.* (1993).

In addition, a method for the successful culture of human ovarian surface epithelium was described by Kruk *et al.* (1990), although this did not include assessment of the responsiveness to exogenous hormonal agents. The potential significance of a model to study human ovarian epithelium was addressed by the authors, who stated that over 85% of human ovarian carcinomas were believed to arise from the surface epithelium. In contrast, animals show very few tumours in this tissue and hence animal tissue models are of little value for the study of this aspect of human disease.

Endometrial cell models

Casslén and Harper (1991)

Method

Endometrial tissue was obtained from uteri removed for benign non-endometrial pathology. Patients were all parous, 26-48 years of age, and showed normal menstrual cycles. A blood sample was obtained intra-operatively for progesterone assessment. Tissue used for culture was obtained by opening the uterus and scraping from the endometrial surface, avoiding the lower uterine segment. After washing, 1 mm pieces were placed in a dissociation solution assisted by occasional mechanical agitation. Subsequent filtration gave good separation between single stromal cells and glands. After filtration, the stromal cell suspension was centrifuged and the cells re-suspended and plated at 10^5 cells/cm² in a serum-free supplemented medium. Glands were plated in collagen (derived from rats) in a modified medium. After one or two days' growth, cultures were stimulated by introduction of varying concentrations of oestradiol. Each experiment was performed in duplicate wells, and samples were stored deep-frozen until assay. Microscopic evaluation of cells involved fixation of cultures with formalin and staining with haematoxylin-eosin or May-Grunwald-Giemsa or by glutaraldehyde and osmium tetroxide processing and subsequent scanning electron microscopic examination. Statistical analysis was by three-way ANOVA and Student-Newman-Keul's test or Student's t-test for paired observations.

Endpoints/data interpretation

PDG_{2α}: analysed by radioimmunoassay and results presented as ng/well × 24 hours.

Serum progesterone: analysed by routine radioimmunoassay; used to define luteal phase of donor.

Histopathological assessment: by phase-contrast light microscopy or scanning electron microscopy.

The cultures had similar cellular characteristics to the expected *in vivo* appearance and the collagen coating of the wells was necessary for efficient growth. This latter point was attributed to collagen

providing a surface basement membrane substitute. Measurement of PDG_{2α} levels showed secretory-[ed?] to have higher levels than proliferative cultures, and showed a clear dose-response relationship to oestradiol. This confirmed the hormonal sensitivity of the endometrial epithelial cells when cultured in serum-free media, and it was felt that this methodology would permit assay for the effects of various drugs on human endometrium. It was suggested that further optimisation of culture medium might be possible to enable longer-term culture.

Markiewicz *et al.* (1993)

Method

Histologically normal endometrium was obtained from patients undergoing endometrial biopsy or hysterectomy. Tissue was subsequently cut into small fragments and placed on lens paper resting on stainless steel grids within a culture dish. The tissue was partially covered with a serum containing medium, pre-treated with dextran-coated charcoal. Randomised fragments of the tissue were fixed in formalin for histological dating. Following a 24-hour initial incubation period, the medium was replaced with one supplemented with either oestradiol (E₂), equol or 4-hydroxytamoxifen (OHTam) alone or in combination. After a further 24-hour period, the medium from each dish was collected, centrifuged and stored deep-frozen. Tissue was recovered, washed, homogenised and analysed for protein content. Levels of PDG_{2α} in unextracted culture medium were also measured. Statistical analysis of differences were estimated by Student's paired t-test.

Endpoints/data interpretation

PDG_{2α}: analysed by radioimmunoassay in duplicate at two dilutions from two dishes for each experimental condition.

Measurement of PDG_{2α} showed a clear increase in response to oestradiol or equal exposure, and the response of the system could be blocked by co-administration of the anti-oestrogen OHTam. It was suggested that this *in vitro* bioassay would be suitable for testing oestrogenic activities of synthetic and naturally occurring chemicals, and that it would provide a more direct alternative to using oestrogen receptor binding assays or experimental animals.

Ovarian surface epithelium (Kruk *et al.*, 1990)

Method

Biopsy specimens were obtained from women between 20 and 62 years of age who were undergoing surgery for non-malignant gynaecological disorders. The specimens were collected aseptically and transported to the laboratory in medium containing fetal bovine calf serum.

Two methods of establishing ovarian surface epithelium cells in culture were investigated. In the first, tissue was taken, trimmed of stroma and cut into explants of approximately 1-2 cm³. These were then placed in culture dishes and held in place by cover slips and cloning cylinders. In the second method, the entire surface of the sample was washed and then held surface down over a culture dish and the ovarian surface scraped firmly two or three times by a rubber scraper attached to a glass rod. The sheets of cells thus formed were rinsed into culture dishes. For either collection method, cultures were incubated at 37°C for 48 hours in 5% CO₂/air. Subsequently the medium was changed as necessary.

The feasibility of storage in liquid nitrogen was investigated, with cells being frozen by standard tissue culture methods either as uncultured epithelium sheets following detachment from the ovarian surface or after a few days' growth on plastic strips.

Endpoints/data interpretation

The scrape method of sample taking was found to be significantly more successful than the explant method in establishing cultures. It also resulted in a purer culture of epithelium cells. Storage in liquid nitrogen (without enzymatic dissociation) was shown not to affect the subsequent proliferative capacity or maintainability of the cells after thawing.

This paper focused on the development of a culture system for human ovarian epithelium rather than for use as a chemical screening model. However, it would be interesting to assess the responsiveness of such cells to sex hormone-disruptive chemicals. The procedures developed enabled the long-term storage of cells (deep-frozen), followed by successful culture when required. This indicates that problems relating to the availability of human primary tissues might be ameliorated by improved storage techniques.

HUMAN CELL LINE SCREENS

Introduction

In addition to the test systems detailed above that employ primary cells from humans, detailed above, a range of other models have been developed that are based upon established human cell lines and have differing regimens and endpoints. Cell lines used include but are not limited to MCF-7, Ishikawa, HeLa and T47D. MCF-7 and Ishikawa cells were reviewed in detail earlier. The remaining cell lines are presented below. McLachlan (1993) suggested a functional approach to detect biologically active xenobiotics (including environmental oestrogens) in which chemicals are defined by their function rather than by their chemistry. It was suggested that this should include human cells transfected with constructs containing a specific receptor and a reporter gene for the receptor, and would allow the assay of not only ligand-receptor binding but also receptor occupancy of the response element and gene activation. Suggested receptors included the oestrogen, progesterone, androgen, glucocorticoid, retinoid and thyroid receptors. Using this approach would allow some careful biological generalisations to be attempted, although it would not provide a complete toxicological profile of a chemical or replace full animal testing.

MDA-MB-231 CL10A cancer cell line

Introduction

Jaing *et al.* (1993) investigated structure-activity relationships for the oestrogen receptor (ER) using stable transfectants derived from an ER-negative breast cancer line, MDA-MB-231 CL10A, to evaluate the effect of a point mutation in the ligand-binding domain of the ER on the pharmacology of the anti-oestrogens tamoxifen, RU39411 and Keoxifenein. Previous studies had focused on ligand structure to establish a model for oestrogenic and anti-oestrogenic action at the binding site of the steroid receptor. The chemicals were tested in the presence and absence of oestradiol (E_2) for the effect on cell growth in cells which expressed the wild type ER_{400 gly} (S30) or a mutant ER_{400 val} (ML α 2H). A control antisense-ER transfectant which did not express ER protein was also included in the study.

Method

Transfectants were maintained in phenol red-free, minimal essential medium supplemented with fetal calf serum (stripped using dextran-coated charcoal). Cells were plated out and maintained in oestrogen-free medium for two days before exposure to vehicle control (0.1% ethanol) or test compound in absolute ethanol. Cells were exposed for eight days, with medium changes every two days. The medium was then removed and the cells lysed by sonication. Total DNA concentrations were determined using Hoechst dye 33258; this was considered to act as a marker for proliferation and growth.

Subsequent development

de Cupis *et al.* (1995) conducted a comparative study using the MDA-MB-321 cell line together with four other lines to compare the relative potencies of two non-steroidal pure anti-oestrogens and the anti-oestrogen tamoxifen. In this study, a number of endpoints were investigated, including cell proliferation as assessed by MTT colourimetric assay, secretion of insulin-like growth factor (IGF-1) assessed by radioimmunoassay, IGF-1 mRNA synthesis assessed by reverse-transcription polymerase chain reaction, together with ligand blot and binding studies. Because this cell line was used in the basic ER-negative form, it was found to be unresponsive to treatment. This study will therefore not be considered further.

Endpoints/data interpretation

Jaing *et al.* (1993):

Total DNA concentration: determined using Hoechst dye 33258. Anti-oestrogenic activity was defined as extent of growth inhibition in ER transfects.

E₂ inhibited the growth of the transfectants S30 and ML α 2H. The ML α 2H cells were found to be 10 to 100 times less sensitive to oestrogens or anti-oestrogens than the S30 form. Keoxifene, an anti-oestrogen with high affinity for ER, displayed anti-oestrogenic activities in both ER transfectants. However, the action of the steroidal anti-oestrogen RU39411 was altered in the ML α 2H cells. The Val₄₀₀ mutation resulted in increased oestrogenic activity of some anti-oestrogens. It was suggested that this might explain the outgrowth of some ER-positive breast cancer cells and some of the reasons which underlie resistance to tamoxifen therapy. None of the cells transfected with the antisense cDNA were affected by any of the chemicals.

T-47D cell lines

Introduction

Welshons *et al.* (1987) evaluated the oestrogenic and anti-oestrogenic activity of two phenolic lignans, enterolactone and enterodiol, and a phytoestrogen, equol, using a human breast cancer cell line, T47D. Phenolic lignans are synthesised from plant-derived lignans by intestinal microflora and are excreted in urine. Similarly, equol is a known phytoestrogen found in urine. Oestradiol was used as a positive control for comparative purposes.

Method

T47D cells were maintained in RPMI 16 40 medium, supplemented with insulin and fetal calf serum. Cells were transferred to medium containing serum (stripped using dextran-coated charcoal) without phenol red for nine days before use. Cells were subsequently plated into wells and allowed to attach for two days before exposure to the appropriate test compound solutions for six days. Progesterone receptor levels were measured in attached cells by whole cell uptake of [³H]R5020 with dexamethasone addition to reduce binding to glucocorticoid receptors. Cell growth was assessed by measurement of DNA levels in each culture well by sonication of the cells, followed by fluorometric measurement.

Subsequent development

Watanabe *et al.* (1990) used several cell lines including T47-D to determine their sensitivity to hormonal agents using a "MINI" radionucleotide precursor incorporation assay (Section 0). Effects of two anti-oestrogenic compounds, tamoxifen and toremifene, along with those of oestradiol on thymidine incorporation were studied as an indication of cell proliferation. Cells were cultured in RPMI 1640 medium supplemented with fetal calf serum and maintained at 37°C in a humidified CO₂-rich atmosphere. Cells were exposed to the test chemicals for 96 hours, after which incorporation of tritiated thymidine was

assessed using liquid scintillation counting. DNA extraction was achieved by precipitation, and centrifugation was followed by fluorometric determination. Cytosolic concentrations of ER and progesterone receptors were determined using commercial enzyme immunoassay kits. Protein concentrations were determined using the Coomassie brilliant blue method.

Endpoints/data interpretation

Welshons *et al.* (1987):

Progesterone receptor stimulation: determined by a competitive binding assay.

DNA level/well: determined fluorometrically and used to assess the degree of oestrogen mediated cell proliferation.

Enterolactone, enterodiol and equol were weakly oestrogenic in comparison to oestradiol. Half-maximal responses were approximately 10^{-11} M for oestradiol, 10^{-7} M for equol and 10^{-5} M for enterolactone. Enterolactone was thus approximately one thenth as potent as oestradiol. The growth of T47-D cells was found to be stimulated in a concentration-dependent manner between 1 and 10 μ M of enterolactone; however, concentrations of 10 M inhibited cell growth, probably through a non-oestrogenic mechanism suggested as peculiar to breast cancer cell lines.

Watanabe *et al.* (1990):

Thymidine incorporation: determined by liquid scintillation count.

Hormone receptor concentration: determined by enzyme immunoassay, expressed relative to protein concentration.

DNA level: determined fluorometrically.

Protein concentration: determined using the Coomassie brilliant blue method.

Thymidine incorporation showed a dosage-related increase with oestradiol treatment, although the increment was only 2.3-fold. Nonetheless, this was considered to show a growth-stimulatory effect of E_2 . Thymidine incorporation was suppressed to 75% or 10% of this by 10^{-7} M and 10^{-5} M tamoxifen, respectively. Suppression of thymidine incorporation by toremifene was not observed at concentrations lower than 10^{-5} M, and dose-response was poor with no greater than 15-20% thymidine uptake inhibition occurring.

BT-20 and STT-11 cell line systems

In the same paper where they described the use of T47-D cells to investigate two anti-oestrogenic compounds, tamoxifen and toremifene, and oestradiol, Watanabe *et al.* (1990) used two ER-poor cell lines: BT-20 (an established ER-deficient line) and STT-11 (a newly established line). Unsurprisingly, the low ER expression resulted in no discernible activity being seen for these cells. No further consideration will be given to them other than to note that the absence of ERs in these cells indicates that they might be of use should controlled levels of receptor expression be required through the use of transfection techniques.

HeLa cell lines

Introduction

Berry *et al.* (1990) attempted to elucidate the cause of differences in *in vivo* activity of two anti-oestrogens, 4-hydroxytamoxifen and ICI-164,384, despite both appearing to act antagonistically to oestrogen. 4-hydroxytamoxifen may act as a partial agonist, depending upon the tissue and the response examined, whereas ICI-164,384 acts only as a pure antagonist. They suggested that this might reflect

differences in interaction with two transcriptional activation domains of the ER. The effects of these chemicals were determined in transiently-transfected HeLa cells which are otherwise devoid of detectable levels of ER.

Method

Expression vectors encoding either the whole hER (HEO), an N-terminally truncated ER containing a transcriptional activation factor, TAF-2 (HE19), and a C-terminally truncated ER containing TAF-1 (HE15) were transfected together with various oestrogen-sensitive reporter genes (ERE-tk-CAT, vit-tk-CAT, 17M/ERE-G.CAT and pS2-CAT) which have complex ER-inducible promoter regions containing several upstream elements of either thymidine kinase (tk), globin (G) or pS2 gene promoters in conjunction with a TATA box. An ERE-TATA-CAT was also used which is a minimal promoter composed of an ERE inserted upstream of the TATA region of the adenovirus-2 major late promoter (Ad2MLP). In addition, a reference plasmid encoding for bacterial β -galactosidase was co-transfected to enable correction for transfection efficiency.

HeLa cells were maintained in DMEM [ed?], supplemented with fetal calf serum (stripped using dextran-coated charcoal). At 50% confluence, cells were transfected using calcium phosphate co-precipitation. Test chemicals were added one hour post transcription. Where appropriate, calcium phosphate precipitate was removed after 18-20 hours, rinsed with DMEM, and replaced with fresh medium containing test ligand or vehicle. After a further 18 to 24 hours, cells were harvested, lysed, and extracts centrifuged to remove cell debris and then assayed for β -galactosidase activity. Extracts were also assessed for CAT activity, using [14 C]chloramphenicol and acetylcoenzyme A, by thin layer chromatography, autoradiography and quantitation by liquid scintillation count.

Subsequent development

Crombie *et al.* (1994) described the use of a cell-based transfection system using the chloramphenicol acyl transferase (CAT) reporter gene to determine hormonal specificity for promotion of the brain isoenzyme of creatinine kinase (CKB) found in large concentrations in the uterus. HeLa cells contain a low level of glucocorticoid receptors but do not contain endogenous receptors for oestrogen, progesterone, androgen or vitamin D, which can be exploited in transfection studies because this enables very low levels of activity to be detected because of the low background activity.

HeLa cells were co-transfected with different intracellular receptor expression plasmids and a CKB reporter construct was then cultured in DMEM without phenol red, supplemented with fetal calf serum. Transfected expression plasmids for the various receptors studied including oestrogen (pKCR-ER), progesterone (pRST7hPRB), androgen (pRShAR), glucocorticoid and vitamin D (pRShVDR) receptors. To adjust for differences in transfection efficiency, cells were co-transfected with a β -galactosidase expression plasmid. Fourteen to 16 hours post transfection, the medium was changed and various ligands at a concentration of 10^{-7} M or vehicle control were added. Cells were harvested 24 hours later and extracts were prepared for CAT and β -galactosidase assay. To increase sensitivity, chlorophenol red β -galactoside was used as a substrate for the β -galactosidase assay. CAT activity was normalised by expressing the percentage conversion of chloramphenicol, divided by the β -galactosidase activity. Fold stimulation was taken to be the ratio of CAT activity in the presence of hormone to that without hormone.

Endpoints/data interpretation

Berry *et al.* (1990):

β -galactosidase activity: determined by measuring the reaction product of *o*-nitrophenol- β -D-galactopyranoside; used to correct for transfection efficiency.

CAT activity: by liquid scintillation count; expressed as fold-stimulation of transcription relative to the values obtained with control cells not treated with oestradiol (E₂) or oestrogens.

The authors commented that the oestrogenic effect of 4-hydroxytamoxifen on the stimulation of transcription by hER was promoter context dependent. In the HeLa cell line, 4-hydroxytamoxifen had very little agonistic effect except in the case of the pS2 gene promoter. Because the hER TAF-2 cannot be induced by this chemical, the agonistic effect was attributed to TAF-1, whose activity was insensitive to 4-hydroxytamoxifen. It was suggested that this could account for the tamoxifen agonistic effects which occur *in vivo* and are dependent on the tissue, cell type and parameter studied. Variations in tamoxifen agonism *in vivo* would depend on the ability of the hER activation function, TAF-1, to stimulate transcription in particular tissues or cells from specific oestrogen responsive promoters. The pure antioestrogenicity of ICI-164,384 would result from a complete inability to induce transcriptional activity of the hER even under promoter context conditions in which TAF-1 is active.

Crombie *et al.* (1994):

CAT Activity: expressed as percentage conversion of chloramphenicol to acetylated derivative.

Only the ER was able to activate the CKB promoter in a hormone dependent manner, with a level of stimulation approximately 100 times higher in the presence of 17 β -oestradiol than in its absence. Progesterone and dihydroxytestosterone, in conjunction with their relevant receptors, had little effect on the CKB promoter, whereas liganded glucocorticoid and vitamin D receptors elicited very weak activation (1.6 and 1.7-fold increases, respectively). Activation of CKB by oestrogen and its receptor was completely blocked by the antagonist, ICI-164,384, which itself had no agonistic effect. The conclusion drawn from these findings was that the hormonal specificity of the rat CKB gene was restricted to oestrogen. When both progesterone and oestrogen receptor were transfected together to investigate the possibility of competition effects, neither progesterone nor its antagonist, RU 486, had any effect on oestrogen-induced CKB promoter activation.

ANIMAL TISSUES AND PRIMARY CELL SCREENS

Introduction

In addition to the *in vitro* systems employing either human primary or established cell lines (Sections 0 and 0, respectively) a number of models using primary cells, tissues or organs of animal origin have been shown to be capable of responding to oestrogenic or anti-oestrogenic chemicals. As such, they represent potential alternatives to the use of human-derived tissue.

Rat uterine culture

Kassis *et al.* (1984) reported on the use of *in vitro* cultures of uterine cells to oestrogenic stimulation and the responsiveness and sensitivity of various endpoints.

Chemicals investigated included 17 β -oestradiol, progesterone, testosterone and dexamethasone.

Method

Immature female rats (21 days of age) were decapitated and the uteri removed and stripped of mesentery. Uterine cells were dispersed in Hank's Buffered Saline by mincing with scissors and mixing with collagenase and DNAase. After one hour, the mixture was centrifuged at 300 g for five minutes, the supernatant discarded, and tissue re-suspended in a pancreatin containing solution. Following a further hour's stirring and subsequent centrifugation and separation, cells were processed to provide single or small clumps of cells. Cells were then cultured in DMEM including dextran-coated charcoal. Cultures were held in dishes incubated at 37°C in an atmosphere of 95% air, 5% CO₂. Media were changed every third day until treatment was started by inclusion of the appropriate test material in the medium. Thereafter, media were changed daily. The following endpoints were studied at the end of the relevant exposure periods.

To monitor the synthesis of proteins by the cultured cells, they were rinsed to remove the culture medium, followed by incubating for six hours in a Ham's medium containing [³⁵S]methionine. After labelling, cells were placed on ice and the medium centrifuged at 5600 g for one minute. Cells were then rinsed and collected in a sodium dodecyl sulfate sample buffer. For SDS-gel electrophoresis, medium samples as well as the cells were analysed. The acrylamide slab gels used were processed for fluorography and exposed to film. In addition, autoradiography was used. Films were scanned using a densitometer. Total protein levels were measured using the method of Bradford (1976) and DNA by the method of Burton (1956).

The assay for progesterone receptor levels involved placing cells on ice following treatment, rinsing, and then scraping into TDG buffer. Cells were then repeatedly drawn through a needle in order to break them. Following centrifugation, the pooled supernatant from four cultures was further centrifuged at 130,000 g for 45 minutes and then co-incubated with [³H]progesterone with or without a 200-fold excess of unlabelled progesterone or unlabelled R5020 (these materials were found to give identical competition and therefore only the unlabelled progesterone was generally used). Assays were performed in triplicate. The method used for oestrogen receptor level assay was generally similar to the previous procedure, with the exception of differences in buffers and the use of [³H]oestradiol and unlabelled oestradiol. For saturation analysis, cells were treated with a range of concentrations of labelled oestradiol with or without a fixed amount of unlabelled oestradiol for one hour at 37°C, followed by rinsing and breakdown of the cells and derivation of the supernatant, as previously described.

Subsequent development

Galand *et al.* (1987): In addition to the *in vivo* work described in Section Uterine biochemistry receptor binding, these authors investigated the cGMP and "induced protein" responses of the uterus to oestrogenic stimulation using an *in vitro* model.

Uterine horns were taken from immature female Wistar rats and incubated at 37°C. cGMP was assessed after incubation in Krebs-Ringer bicarbonate medium for two hours. For the treated groups, this medium was supplemented with 17 β -oestradiol or *o,p'*- or *p,p'*-DDT. Group sizes of 25 to 27 were used. Uteri were subsequently heated in a boiling water bath with distilled water and the cGMP level assessed using a method by Steiner *et al.*, 1972 (*J. Biol. Chem.* 247, 116). For the induced-protein assay, uteri were

incubated for three hours in the incubation medium with or without test materials being added. For the final two hours of incubation, [³⁵S]methionine was added to the incubation medium. Cytosols were then prepared and fractionated using DEAE-cellulose chromatography and SDS-polyacrylamide gel electrophoresis. Labelling intensity in the protein bands was compared by fluorography.

Endpoints/data interpretation

Kassis *et al.* (1984):

Protein identification: by SDS-gel electrophoresis.

Progesterone receptor levels: by radioassay.

Oestrogen receptor levels: by radioassay.

Most cells in the culture were of fibroblastic appearance. Oestrogen-binding ability was assessed after three days' culture and was found to show a response curve saturable at approximately 1 nM of oestradiol. Further work showed that receptor levels increased by day 7 of culture and then maintained this level till cell senescence and death at day 12 onwards. Exposure of cells to oestradiol from days 3 or 6 was shown to result in a dosage-related increase in binding of labelled progesterone three days after first exposure; DNA values were unaffected. Exposing cells to progesterone, testosterone or dexamethasone did not elicit any response. Investigation of the protein profile of the cells in response to oestradiol showed an elevation of the level of a 130K protein following exposure to oestrogen; dosage relationship was found. Of the other chemicals examined, only high levels of testosterone were found to elicit any such response, although high levels of dexamethasone induced another protein of a similar molecular weight.

Galand *et al.* (1987):

cGMP levels: using the method of Steiner *et al.*, 1972 (see Section Subsequent Development).

Induced protein levels: assessed by fluroscopy.

Elevation of cGMP levels was reported following incubation for two hours with either form of DDT investigated, although the effect was not as great as that elicited by the oestradiol. The similarity of response between the DDT isomers differed from that seen *in vivo*, where the *pp* form was shown to have lower activity. Oestradiol and *o,p'*-DDT were also found to induce formation of the same protein.

Rat pituitary culture

Lieberman *et al.* (1978) described a method for the culture of rat pituitary cells and subsequent measurement of prolactin synthesis rates in response to treatment with a number of test chemicals. These included 17 β -oestradiol, diethylstilboestrol, progesterone, testosterone, 17 β -hydroxy-5 α -androstan-3-one and corticosterone.

Method

Anterior pituitaries were dissected from 18-day-old female rats and the cells dispersed using a collagenase; viability was assessed using trypan blue exclusion. Cells were subsequently cultured in flasks using a medium containing sera which had first been treated with dextran-coated charcoal to remove free steroids. The appropriate test chemical, dissolved in ethanol, was added at various concentrations to the cultures. Controls were also established. Media were changed every two to three days during the study. Cultures were terminated at intervals during a seven-day period in order to determine the time course of effects. At the end of the appropriate exposure period, flasks were rinsed with leucine-free Earle's minimal essential medium and incubated for 60 minutes with medium containing either [¹⁴C]leucine or [³H]leucine. Cells were removed, homogenised and deep-frozen. Methodology varied, depending on whether electrophoresis on polyacrylamide gel or quantitation using immunoprecipitation techniques was

intended. The extracts of the cultures were polyacrylamide gel electrophoresed, whilst immunoprecipitation was achieved with antisera to prolactin in the presence of a known quantity of [¹⁴C]-labelled prolactin. After subsequent incubation, radioactivity was assessed by scintillation count. Mean values were calculated from four cultures per treatment, and statistical analysis was by analysis of variance and Students t-test.

Subsequent development

Lieberman *et al.* (1983) used a similar model system to investigate the effects of anti-oestrogens on pituitary cells stimulated to produce prolactin by treatment with oestradiol. Tamoxifen or a number of its metabolites were added to cultures either alone or in combination with oestradiol. Oestradiol alone and untreated control cultures were also established. Log concentration against percentage prolactin synthesis was plotted to establish the effects of the various treatment regimens. In a separate experiment, inhibition of [³H]oestradiol binding by the anti-oestrogens was studied for rat pituitary or uterus. In these studies, homogenates of the relevant tissue were centrifuged and the cytosol supernatant removed. Aliquots of the cytosol were then incubated for 30 minutes with different concentrations of oestradiol and a known quantity of tritiated oestradiol. After cooling in ice water, dextran-coated charcoal was added and, after centrifugation, the supernatant was decanted and radioactivity measured by a standard scintillation count. Parallel incubations using tritiated oestradiol with or without diethylstilboestrol allowed determination of the specific binding of the oestradiol. From these data relative binding affinities were calculated.

Jordan *et al.* (1986) used a pituitary cell culture system to investigate the effect of a series of triphenylbut-1-enes on prolactin synthesis by rat pituitary cells and also established their relative binding affinities for the oestrogen receptor. The procedures used for pituitary cell culture and endpoint measurements were generally similar to those of Lieberman *et al.*, whilst relative binding affinities for the oestrogen receptor were studied using rat uterine cytosol extracts.

Endpoints/data interpretation

Electrophoretic profile: to identify proteins showing increased levels.

Radioactive counts: quantitation of prolactin levels by measurement of radioactivity incorporation using scintillation count.

Relative binding affinity: calculated by Lieberman *et al.* (1983) for the oestrogen receptor.

In the study by Lieberman *et al.* (1978), oestradiol specifically stimulated prolactin synthesis without a significant change in the profile for other cellular proteins. The effect was dosage-related from 10⁻¹¹ M to a near maximum at 10⁻⁸ M. Similar effects, although at different levels, were found for oestradiol, oestriol and diethylstilboestrol. In contrast, the androgens, progesterone and corticosterone had only minimal effects. Examination of the time course of the changes showed a significant increase by three days that continued to increase throughout the seven-day experimental period. Lieberman *et al.* (1983) demonstrated that the pituitary cell culture system was sensitive to the presence of anti-oestrogens, which caused an inhibition of prolactin synthesis. Separate studies on cytosolic extracts of rat pituitary and uterine tissue demonstrated that these chemicals inhibit binding of oestradiol to the oestrogen receptor. The study by Jordan *et al.* (1986) further demonstrated the responsiveness of the pituitary cell culture system to oestrogenic and anti-oestrogenic modulation, and the relationship of the responses seen to the chemicals' relative binding affinities to the oestrogen receptor. This system permitted these workers to further investigate receptor-binding models.

Mouse Leydig cell culture

Bilinski (1986) reported on the responsiveness of an *in vitro* mouse testicular cell culture system to 17 β -oestradiol exposure. This system used several endpoints to monitor Leydig cell functionality.

Method

Two-month-old male Swiss mice were killed by cervical dislocation. The testes were dissected out and placed in Petri dishes containing physiological buffered saline and a Leydig cell suspension were derived. Ten males were used to provide tissue for each culture.

Cultures were divided into treated and control groups; the medium for the treated groups included either oestradiol or oestradiol in combination with leutanising hormone. Cultures were maintained in medium 199 with the addition of 10% calf serum. Media were replaced every second day and the used portions of the medium were stored deep-frozen for radioimmunoassay for androgen levels. This used [1,2,6,7-³H] testosterone as a tracer and an antibody raised in rabbits against testosterone 3-o-carboxymethyloxime-bovin serum albumin. Additional endpoints investigated included the examination of cell morphology following staining of cultured cells by May-Grunwald Giemsa techniques. Determination of $\Delta^5,3\beta$ -hydroxysteroid dehydrogenase and 17 β -hydroxysteroid dehydrogenase levels was by histochemical analysis using dehydroepiandrosterone and testosterone as substrates, NAD as a co-factor, and nitro blue tetrazolium. Response was scored on a four-point scale.

Endpoints/data interpretation

Androgen levels: by radioimmunoassay.

Enzymatic activity: assessed by histochemical techniques for $\Delta^5,3\beta$ -hydroxysteroid dehydrogenase and 17 β -hydroxysteroid dehydrogenase.

Cellular morphology: by light microscopy.

Cellular attachment to the glass of the culture containers occurred from day 2 of culture and by day 3 a homogeneous monolayer was formed. The morphological appearance of the cells was shown to change with the age of the culture, but was found not to be influenced by oestradiol treatment. Enzyme activity could be detected from the second day of culture, although there was subsequently a decline in levels that was associated with cellular ageing and confluence of growth. Treatment with oestradiol or oestradiol and lutenising hormone in combination resulted in a lowering of dehydrogenase activities. Marked differences in androgenic secretion by the cultured Leydig cells were seen in response to the various treatment regimens. Control levels increased from day 2 to day 4 but decreased thereafter. Oestrogenic exposure reduced the androgen levels, whilst the combined treatment resulted in an even more marked depression.

ANIMAL CELL LINE SCREENS

Transfection techniques have been used to insert hormonal receptor structures, linked to reporter gene constructs, in a number of cell types which might or might not constitutively express such receptors. A number of such models that have been designed to study oestrogenic interactions are reviewed below.

Transfected chicken embryo fibroblast cells (CEFS)

Introduction

In 1990, Berry *et al.* published methods which included the use of chicken embryo fibroblast cells (CEFs) to investigate the agonistic activity of the anti-oestrogenic compounds 4-hydroxy-tamoxifen (OHT) and ICI-164,384.

CEF cells were transfected with reporter genes expressing truncated or chimeric human oestrogen receptors (hER) with either of two independent hER transcriptional activation functions, TAF-1 or TAF-2. In this case, the reporter gene construct coded for a chloramphenicol acyl transferase (CAT) with the addition of β -galactosidase as an internal control standard to account for differences in transfection efficiency.

Method

Chicken embryo fibroblast cells were prepared from 9- to 11-day-old embryos. Cells were maintained in DMEM and transfected with either vit-tk-CAT, ERE-tk-CAT, ERE-TATA-CAT, 17M/ERE-G.CAT, pS2-CAT or 17M2-G.CAT as reporter genes, together with either HEO, HEGO, HE19, HE19G, HE15 or GAL-ER(AB) or GAL-ER(EF) as activator genes. Transfections also contained the reference plasmid pCH110 (expressing bacterial β -galactosidase, and Bluescribe M13+ DNA (BSM+) as carrier DNA).

Ethanol (vehicle control), oestradiol, 4-hydroxytamoxifen or ICI-164,384, were added one hour post transfection. Where appropriate, the calcium phosphate precipitate was removed after 18 to 20 hours, cells were rinsed in DMEM, and fresh medium containing the appropriate test ligand was added. After an additional 18 to 20 hours, cells were rinsed and harvested, lysed and centrifuged to remove cell debris. Each extract was assayed for β -galactosidase activity. CAT was assayed over one hour in the presence of [¹⁴C]chloramphenicol and acetyl coenzyme A. Acetylated and non-acetylated forms of labelled chloramphenicol were separated by TLC and autoradiographed before being excised and quantitated by liquid scintillation counting.

Subsequent development

White *et al.* (1994b) published an assessment of the potential oestrogenic activity of a range of alkylphenolic compounds using a number of test systems including CEF cells.

CEF cells were cultured in DMEM and used with and without inclusion of an oestrogen response element (ERE). A transfected DNA reporter plasmid, pEREBLCAT, was used together with an internal control plasmid, pj3 luciferase and pJ3 Φ . pEREBLCAT contained an ERE derived from a vitellogenin A2 promoter upstream of the herpes simplex viral thymidine kinase gene promoter linked to the CAT reporter gene. Cells were maintained with no hormone, oestradiol, one of the alkylphenolic chemicals, or an anti-oestrogen over a 48 hour exposure period. Cells were then harvested and assayed for luciferase and CAT activity. Experiments were carried out in duplicate and replicated at least twice.

Endpoints/data interpretation

Berry *et al.* (1990):

β -Galactosidase: to normalise for differences in transfection efficiency.

CAT: expressed as the fold-stimulation of transcription relative to the values obtained with HEO in control cells not treated with oestradiol (E2) or anti-oestrogens.

The oestrogenic effect of OHT on stimulation of transcription by the human ER was both cell type and promoter context-dependent. In CEF, 4-hydroxytamoxifen acted as an efficient agonist irrespective of promoter context. It appears that the transcriptional activation function TAF-2 cannot be induced by OHT. The agonistic effect of OHT is ascribed to TAF-1, whose activity is insensitive to OHT. TAF-1 stimulates transcription efficiently from all oestrogen-responsive promoters in CEF. On this basis, it was proposed that OHT acted as an oestrogen agonist whenever it promoted binding of the hER to a target gene promoter from which transcription could be activated by TAF-1 on its own. Conversely, pure antagonistic activity would occur whenever the activation of a given promoter was fully dependent on TAF-2 activity.

White *et al.* (1994b):

Luciferase: used to correct for differences in transfection efficiency.

CAT: expressed as the fold induction of the reporter in the absence and presence of the chemical.

It was shown that transcriptional stimulation was dependent on the presence of co-transfected receptors and confirmed that the activity of the chemicals studied was mediated via the ER. It was possible to detect differential activities for the chemicals at concentrations ranging from 10^{-8} to 10^{-5} M. In addition, the presence of an anti-oestrogen was associated with inhibition of response.

Transformed mouse L-cells

Introduction

Mayr *et al.* (1992) assessed the oestrogenic potential of a number of myco- and phyto-oestrogens using LeC-9 cells. These were a genetically transformed cell clone derived from mouse L-cells constructed using transfection of a reporter gene. Hormone-like activity was measured by the expression of the CAT gene under the control of an ERE. Toxic effects on cell viability were monitored by expression of a second oestrogen-insensitive reporter gene coding for a bacterial β -galactosidase.

Method

Le42 cells were maintained in DMEM. Five days before transfection, this was replaced with an oestrogen-free medium (stripped using dextran-coated charcoal). Le42 cells were transfected with the following recombinant plasmids: pA2(-331/-87)tk-CAT (CAT gene construct); pH β APr-1- β GAL (β -galactosidase gene construct) and pTKcos2 (containing a thymidine kinase gene and its promoter, necessary for HAT selection). These were transfected using a calcium phosphate precipitation technique. After one day, cells were suspended in HAT medium for isolation of stably transfected cells. HAT selection continued for three weeks until cell clones were visible.

Compounds tested were 17 β -oestradiol (positive control), zearalenone, coumestrol, genistein, daidzein, biochanin A, formononetin, genistin, daidzein, tamoxifen, progesterone, testosterone, and a pure anti-oestrogen, 1(aminoalkyl)-5-hydroxy-2-(4-hydroxyphenyl)indols.

CAT activity was determined using a reaction mixture containing cell lysate, buffer, acetylcoenzyme A, and [14 C]chloramphenicol. Chloramphenicol and enzymatically acetylated derivatives were extracted with ethylacetate and separated by thin layer chromatography. CAT activity (as percentage of acetylated substrate) was then calculated. β -GAL activity was determined using *o*-nitrophenol- β -D-galactopyranoside as substrate.

Endpoints/data interpretation

CAT activity: expressed as percentage of acetylated substrate. The relative oestrogenic activity was calculated against 17 β -oestradiol.

β -GAL activity: determined from the reaction product of *o*-nitrophenyl- β -D-galactopyranosid, at 420 nm.

ER-mediated activity could be shown for a wide range of myco- and phyto-oestrogenic chemicals. Differential activities could be detected from 10^{-10} to 10^{-5} M.

ANDROGEN-RELATED

HUMAN CELL LINES

Saos-2 and U2-OS human osteoblast cell lines

Introduction

Orwoll *et al.* (1991) described the potential role of sex steroids and, in particular, androgens in the growth and differentiation of osteoblastic cells. In studies they examined two human osteoblast-like cell lines, Saos-2 and U2-OS, for the presence of specific high-affinity androgen-binding receptors. This work should be considered as exploratory, being aimed at identifying hormonally responsive cell types rather than constituting a developed test model.

Method

The two human cell lines of osteoblast phenotype used in the study were both of female origin and were grown in a McCoy's 5a-based medium. Cells were grown to 90-95% confluence before harvesting and storage at -70°C. Androgen receptor quantitation was carried out separately for cytosolic and nuclear fractions. Crude nuclear fractions were processed by salt extraction and, after centrifugation, residual nuclear pellets used for DNA quantitation. Androgen receptors were quantified in the cytosolic and nuclear salt extracts by titration using [³H]R1881. Non-specific binding was established by competitive displacement with excess, unlabelled R1881 to determine specific binding. Triamcinolone acetone was added to prevent binding to progesterone receptors. Bound [³H]R1881 was separated from free radioligand in cytosolic fractions by the addition of a dextran/charcoal slurry whilst, for nuclear preparations, hydroxyapatite was used. Radioactivity was measured by scintillation spectrometry and specific binding estimated by Scatchard analysis.

The interaction of the receptors with a number of different chemicals was also studied; these included dihydroxytestosterone, R1881 and progesterone. The presence of other receptor types, such as oestrogen and progestin, was also investigated. Binding assays were controlled relative to a pool of human prostate tissue having known concentrations of receptor, and compared with renal and human prostatic carcinoma levels.

Endpoints/data interpretation

Androgen receptor binding: determined by liquid scintillation counting and Scatchard analysis.

It was reported that both osteoblast cell lines displayed specific androgen binding sites, with binding activity similar to that of normal prostatic and renal tissue but lower than in human prostate carcinoma. The binding was saturable in the cell lines and in normal tissues. In both cell lines, administration of high concentrations of diethylstilboesterol did not compete for [³H]R1881 binding whereas excess concentrations of unlabelled dihydroxytestosterone and R1881 produced high displacement of the radiolabelled ligand. It was notable that progesterone caused considerable inhibition of androgen binding. No oestrogen receptor immunoreactivity was detected in the two cell lines. However, progestin receptors were clearly detected.

ANIMAL TISSUES AND PRIMARY CELL LINE SCREENS

Mouse embryonic hypothalamic cell culture

Introduction

Beyer *et al.* (1994) described a method for the culture of brain cells taken from mouse embryos to demonstrate changes in aromatase activity following exposure to a number of steroids either alone or in combination. These included 17β -oestradiol, testosterone, progesterone, 17β -oestradiol with progesterone, and testosterone with flutamide.

Method

Balb/c mice were maintained under standard husbandry conditions and mated. Fetuses were removed on day 15 after mating and sexed on the basis of gonadal structure. The hypothalamus and cerebral hemispheres were dissected and the cells dispersed by trypsin incubation and mechanical agitation. Following filtration, the cells were plated in a suitable medium. The appropriate hormone (*i.e.* test chemical) was added at levels similar to those known to occur naturally in fetal rats. In some of the experiments a range of concentrations was examined to permit study of a chemical's dosage-response characteristics. Controls were established as appropriate. Media were changed daily during a culture period of up to six days.

For aromatase activity measurement, cultured cells were physically harvested at the end of the culture period, homogenised, and stored deep-frozen until analysis. Aromatase activity was measured using a $^3\text{H}_2\text{O}$ formation assay in which aliquots of the cellular homogenates were incubated in a microplate with 1β -[^3H]testosterone. The reaction was stopped by the addition of water followed by freezing. The generated $^3\text{H}_2\text{O}$ was separated using sorbent microcolumns and activated charcoal, and unbound $^3\text{H}_2\text{O}$ was measured by scintillation counter.

In some cases, immunocytochemical examination was performed using a specific polyclonal antisera for mouse aromatase. Cultures were fixed with a paraformaldehyde solution. After permeabilisation with saponin, cultures were incubated for 48 hours with the primary antisera for aromatase. Neurones were immunostained with monoclonal MAP II antiserum, and astrological cells and proliferating cells were labelled with a monoclonal antibody to glial fibrillary acidic protein, vimentin and proliferating cell nuclear antigen. Immunoreaction was visualised by biotinylated bridge-antisera and the avidin-biotin-complex method followed by the diaminobenzidine reaction. Counting of aromatase immunoreacting neurones in each well was by visual microscopic examination.

Effects of gender, brain region and chemical exposure on aromatase activity, and number of neurones with aromatase activity, were assessed using five wells per experiment. Statistical analysis of aromatase reactive neurones and aromatase activity was by one-way analysis of variance, followed by Newman-Keuls multiple-range test.

Endpoints/data interpretation

Aromatase activity: measured using a $^3\text{H}_2\text{O}$ formation assay.

Immunohistopathology: by visual count of aromatase-reactive neurones.

Aromatase activity was detected in both hypothalamic and cortical cell cultures. However, in the absence of hormonal stimulation the levels were different for each sex only in the case of the hypothalamus: levels of activity and numbers of aromatase-containing neurones were highest in the male. Treatment with testosterone significantly enhanced levels only in the hypothalamus of both sexes, although a sex difference in degree of response was observed. Co-treatment with the androgenic receptor antagonist

flutamide completely inhibited the stimulatory activity of testosterone. Treatment with the oestrogen or progestagen was without effect on aromatase activity or distribution.

ANIMAL CELL LINE SCREENS

Introduction

Both mammalian and insect cell lines have been used as assay systems for androgenic activity.

Transfected PC12 neuronal cells

Introduction

In 1994, Lustig *et al.* described a method using a transfected neuronal wild type cell line, PC12-WT, derived from a rat phaeochromocytoma. In the presence of nerve growth factor (NGF), the cells become postmitotic and initiate neurite outgrowth. These cells are easily transfected by mammalian expression vectors and do not express any native androgen receptor (AR) mRNA or protein.

Method

PC12-WT cells were grown in DMEM to 50% confluence and were transfected by a calcium phosphate co-precipitation technique. Cells were transfected utilising the expression vector pCMV-AR-neo, which has previously been shown to transactivate the pMSG-CAT reporter gene. The vector consisted of a strong constitutive cytomegalovirus immediate early gene promoter and enhancer, which is known to express in PC12 cells. This was spliced to a polycistronic-DNA coding for human AR, which was in turn fused to a cDNA cassette for the enzyme neomycin aminoglycoside phosphotransferase (*neo*) for selection of transformants. Selection was carried out by addition of G418 (a neomycin analogue) and clones were isolated and expanded in oestrogen-free medium. Final selection was carried out by mRNA and protein analysis with the AR8 clone ultimately being revealed as AR-positive and androgen-responsive. As negative controls for receptor-specific androgen action, two other derivatives of the PC12 cell line, NEO9 and SER8, were also used. These featured constructs lacking a receptor, in the case of NEO9, or coding for the human oestrogen receptor, in the case of SER8.

Cells were exposed to nerve growth factor (NGF) with dihydroxytestosterone (DHT) for two days, after which a number of endpoints were measured. Poly-A RNA extracts from the four clones, plus an exposed hAR positive control, were probed with a [³²P]-labelled cDNA coding for the hAR. Presence of bands indicated the receptor. In the case of negative controls, no hybridisation took place and as a result no receptor indicating bands were detected. Functional receptor protein was assayed, using cytosol extracted from each cell line, which was incubated with [³H]R1881 so as to determine specific and non-specific binding. A baculovirus AR protein was also assayed as a positive control. Binding was quantitated and used to construct Scatchard plots for samples which displayed specific binding. Changes in cell morphology were characterised using a neurone-tracing system. For each cell, total neurite length, neurite branch segment length, neurite field area and somal area were computed. Poly A⁺ RNA was prepared from samples exposed to androgen and NGF, along with the relevant controls. After separation, transfer to a nylon membrane and cross-linking, samples were probed with [³²P]cDNA corresponding to the NGF receptor and constitutively expressed proteins for standardisation. Bands were elucidated by autoradiography.

Endpoints/data interpretation

Northern blotting for AR mRNA: presence of bands indicated presence of receptor.

Cytosolic [³H]R1881 binding: quantitated and used to construct Scatchard plots for samples which displayed specific binding.

Quantitative light microscopy: total neurite length, neurite branch segment length, neurite field area and somal area were computed.

Assessment of androgenic regulation by Northern blotting: bands elucidated by autoradiography and quantitated relative to constitutive expression.

AR8 cells displayed an androgenic, dose-dependent increase in mean neurite length, branch order, and neurite field area. Neurite branch segment length and soma area were not affected. No such changes in cell morphology were observed in the controls after DHT exposure. Assessment of synergistic effects of DHT and NGF by evaluation of regulation of NGF receptor mRNA produced no significant induction. The results indicated that, for cells expressing AR, androgen acts additively with NGF to increase neurite outgrowth but that the effects of androgens are mediated specifically through branching and arborisation.

Insect cell line

Introduction

In addition to the above method employing mammalian cells, sensitivity to androgens has been established by Wong *et al.* (1993) for an insect cell line following transfection with a human androgen receptor (AR). The principal focus of this paper was to study mechanisms of interactions between androgens (AR) and DNA.

Method

The test system was able to show responses to dihydroxytestosterone and various anti-androgenic agents through measurement of AR binding to an AR element (ARE) in the DNA. An insect cell line, Sf9, derived from *Spodoptera frugiperda*, was used and the transfection system comprised a baculovirus, *Autographa californica*, containing a recombinant human AR.

DNA binding of AR to the androgen response element (ARE) was measured by gel mobility shift DNA binding assay. The degree of double-stranded binding in the presence and absence of androgen, other hormones, or anti-androgens was assessed using labelled [α -³²P]dCTP and the Kenow fragment of DNA polymerase. Samples were electrophoresed, dried under vacuum and autoradiographed.

Endpoints/data interpretation

DNA binding of AR: measured by gel mobility shift DNA-binding assay.

A high degree of specificity to the presence of the androgenic hormone was noted and two possible modes of action for different anti-androgens were identified.

CELL-FREE ASSAYS FOR OESTROGENIC, ANDROGENIC OR RELATED ACTIVITIES

RECEPTOR-BINDING STUDIES

Steroid sex hormones are known to regulate gene transcription via receptor binding. Although the hormone-receptor complex is required for transactivation, the mechanisms governing this phenomenon are not fully understood. Available information indicates that an important feature of activation may rest in conformational changes induced in the receptor by ligand binding.

Receptor binding studies are possible because of the high affinity that some active ligands have for their receptor. Consequently, at low concentrations of chemical a high proportion is bound to the receptor compared to the proportion which binds to non-receptor sites. The amount of drug bound can be measured by radiolabelling it and measuring the amount of radioactivity in the bound tissue. Separation of the bound drug from that free in solution is effected by the use of filtration or centrifugation, the former being the method of choice, assuming that the drug does not dissociate significantly during the filtration process or does not bind to the filter.

A series of authors have presented methods which utilise such assay techniques. Determination of the binding characteristics for a number of steroid-type receptors has therefore been carried out for a number of receptor types including oestrogen, progesterone, androgen and Ah. In initial studies described by Jensen and Jacobson (1960) the fate of steroid oestrogens was studied in various target tissues. Much of the work of the intervening years until the late 1980s concentrated very much on endpoints unconnected with sex hormone-disrupter toxicology.

Korach *et al.* (1988); Phillips *et al.* (1990) and Christman *et al.* (1995) described binding studies utilising oestrogen receptors from rat uteri. DeVito *et al.* (1992) and Harper *et al.* (1994) described studies using the steroidal type Aryl-hydroxylase receptor. Botella *et al.* (1987), Cook *et al.* (1992) and Kelce (1995) performed studies using androgen receptors, whilst Phillips (1990) worked on progesterone receptors.

General methodologies consisted of preparation of cytosolic fractions which were either used as extracted or enriched by using ammonium sulfates as a protein precipitant. Affinity or competition assays were performed followed by a Scatchard or computational analysis. Incubations of test compound ranged from 30 minutes to 24 hours.

Receptor binding affinity assays have become a frequent means of comparison with other bioassay systems, attempting to predict a compound's potential for sex hormone-disruption. The major drawback of this approach is that affinity is thought to be a poor predictor of the type of response which may be elicited. High affinity is no indicator of whether a chemical has agonist, antagonist or mixed effects. Comparison of analogous structures to physiological ligands is also not a good predictor of activity; in the case of the steroidal oestrogens, they are relatively ridged, polycyclic structures with pronounced asymmetry, and their binding to the oestrogen receptor reflects a degree of stereo specificity. In contrast, the non-steroidal synthetic oestrogens usually have elements of symmetry and conformational flexibility, which makes it difficult to determine the exact nature of the binding interaction with the receptor and the structure of the active conformer. Korach *et al.* (1988) quoting from Landvatter and Katzenellenbogen (1981).

The whole issue of ascribing high affinity with high activity is contentious: Korach *et al.* (1988) described experimental results which they claimed clearly showed a relationship between receptor binding affinity and biological activity. They also cited results which indicated that PCB compounds that showed

appreciable receptor-binding activity were also active *in vivo* in stimulating uterine weight increases; conversely, weak binders were found to be inactive. These findings may, however, reflect the consideration of a single class of compound. Other authors make no such claims for these techniques.

MICROTUBULE POLYMERISATION

A cell-free method detailed by Metzler and Pfeiffer (1995) used the effect that oestrogenic chemicals have on the polymerisation of microtubules. These are highly labile cytoskeletal structures that are sensitive to specific antimitotic chemicals. It was reported that the well known synthetic oestrogen diethylstilboestrol has effects similar to the antimitotic agent colchicine; these include inhibition of microtubule (MT) polymerisation under cell-free conditions. The aim of the investigation was to establish whether interaction with MTs is a property shared by all oestrogens.

METHOD

The effects of several classes of oestrogens were studied. These included stilbene oestrogens, steroidal oestrogens, phytoestrogens, mycoestrogens and lignans. Test compounds were dissolved in ethanol or DMSO and were added to micro tubule proteins from bovine brain suspended in assembly buffer. Concentrations of the oestrogens varied from 10 to 100µM and the solvent concentration was 2%. The assay mixture was incubated at 37°C for 20 minutes, MT assembly being initiated by the addition of guanosine triphosphate.

ENDPOINTS/DATA INTERPRETATION

Percentage polymerisation: The degree of polymerisation was assessed by measuring the increase in turbidity at 350 nm for 30 minutes. A control incubation (minus test compound) was used as the reference (100% assembly). Depolymerisation was carried out at 4°C to confirm microtubule formation and detect aggregation.

All the stilbene oestrogens tested were reported to cause a 10-40% inhibition of microtubule assembly. Bisphenol A was reported to display high inhibitory activity which was approximately half that of DES. In contrast, it was reported that none of the steroidal oestrogens showed a detectable inhibitory effect on MT polymerisation, nor did any of the phytoestrogens and mycoestrogens.

OESTROGEN-2-HYDROXYLASE (AROMATASE) ACTIVITY

Osawa *et al.* (1993) detailed experiments in which it was found that the microsomal enzyme responsible for oestrogen-2-hydroxylase was a multifunctional enzyme also responsible for aromatase activity. Oestrogen 2-hydroxylation is a major metabolic pathway for oestrogens known to be carried out by the microsomal fraction of human placenta. Aromatase is implicated in the conversion of androgens to oestrogens and has been identified as a useful point of intervention for medicinal endocrine disruption, an example being the treatment of some forms of breast cancer.

METHOD

Initial experiments were concerned with purification of the enzyme complex, and confirmation that the enzyme known to be oestrogen-2-hydroxylase was by the same active site also the enzyme aromatase. The

methods are not detailed in full, as the work was not directly relevant as a test methodology. However, the findings have been subsequently developed by other groups.

SUBSEQUENT DEVELOPMENT

O'Reilly *et al.* (1995) used the aromatase activity of placental microsomes to investigate the inhibitory effect of 7α and 7β -arylaliphatic-substituted androst-4-ene-3, 17-diones, compounds with potential application to the treatment of breast cancer. Placental microsomal fractions were exposed to test compounds for a five minute period before determining aromatase activity.

Inhibition of aromatase activity was determined by following the tritium released as $^3\text{H}_2\text{O}$ from [1β - ^3H]androst-4-ene-3,17-dione and this was used as an index of oestrogen formation. [1β - ^3H]androst-4-ene-3,17-dione and a concentration of inhibitor were pre-incubated at 37°C for five minutes. Buffered placental microsomes were added to the incubation to initiate the assay, which was subsequently terminated after 15 minutes with the addition of trichloromethane. Samples were mixed followed by centrifugation, before CHCl_3 solvent extraction of the aqueous supernatant. Radioactivity of the aqueous phase was determined by liquid scintillation, with the degree of inhibition being determined as a percentage of the control, which was incubated without any inhibitor.

ENDPOINTS/DATA INTERPRETATION

Percentage inhibition of aromatase activity: calculated relative to uninhibited control aromatase activity.

MONOCLONAL ANTIBODY ENZYME IMMUNOASSAY FOR SIMULTANEOUS QUANTITATION OF 2- AND 16α -HYDROXYOESTRONE IN URINE

2- and 16α -hydroxyoestrone are the major metabolites of oestradiol, being hydroxylated at the C-2 or C- 16α position by the action of cytochrome P_{450} mixed-function oxidase, with the metabolites having oestrogen antagonist and agonist activities, respectively. The balance between these competing oxidative pathways determines, in part, the net *in vivo* oestrogenic stimulus. It has been postulated that relatively increased 16α -hydroxylation of oestrogen may be an intermediate biomarker for the risk of developing breast and other oestrogen-dependent cancers. The method evaluated was developed by Klug, Bradlow and Sedkovic (1994) and is an enzyme immunoassay for measurement of the concentrations of these metabolites. Although Radio IAs have previously been reported, determinations are currently mainly measured using gas chromatographic-mass spectroscopy (GC-MS). The authors hoped that a sensitive and specific immunodiagnostic assay to determine the balance between these competing pathways might be used as a routine biomarker for the management of oestrogen-related disease states.

METHOD

These metabolites are present as glucuronide and sulfate conjugates in urine and therefore must be deconjugated by the addition of β -glucuronidase and arylsulfatase. After two hours, these samples were diluted in neutralisation buffer and aliquotted in replicate into the wells of monoclonal antibody-coated anti-2-OHE and anti- 16α -OHE1 EIA plates. This was followed by the addition of aliquots of 2-OHE:alkaline phosphatase, and 16α -OHE1:alkaline phosphatase conjugates were added to the respective plates, and these were incubated for three hours at room temperature. *p*-Nitrophenyl phosphate was added to each plate and the absorbance read kinetically at 420 nm.

ENDPOINTS/DATA INTERPRETATION

Concentration determination: results presented as dose-response curves, with rates (milli-absorbance units/minute) versus concentration of 2-OHE or 16 α -OHE1 (in ng/mL).

The results of the assay for the two compounds were compared with results from the conventional GC-MS method and yielded correlation coefficients of approximately 0.94 for both.

DEVELOPMENT OF A MARKER OF OESTROGENIC EXPOSURE IN HUMAN SERUM

Sonnenschein *et al.* (1995) referred to epidemiological studies suggesting that increased plasma concentrations of “bioavailable” ovarian oestrogens (*i.e.* unbound to plasma proteins) in postmenopausal women correlate positively with incidence of breast cancer. These studies reported conflicting findings suggesting a possible positive correlation between plasma concentrations of DDE, a DDT metabolite, and breast cancer incidence. The presence of these and other oestrogenic compounds in serum may represent cumulative lifelong exposure, with the main risk factor for developing breast cancer being lifelong exposure to oestrogens. Xeno-oestrogens may act cumulatively to induce cell proliferation, which could potentially be used to develop a marker of exposure.

Sonnenschein *et al.* subsequently described a novel procedure to extract and separate xenoestrogens from ovarian oestrogens in human serum, followed by determination of xeno-oestrogen concentration using a bioassay procedure.

METHOD

Human serum prepared from out-of-date blood bank plasma and from blood drawn from volunteers was clarified by centrifuging. Sex steroids were stripped using dextran-coated charcoal at 37°C for one hour. This removed more than 99% of serum sex steroids, as indicated by removal of a [³H]E₂ spike, determined by radioimmunoassay. Xenoestrogens were isolated from samples by multiple organic solvent extraction. Organic phases were pooled, concentrated, and the dissolved compounds eluted by manual perfusion using a normal phase silica cartridge and three solvents of increasing polarity. The least polar fraction was acid cleaned before drying; other fractions were simply dried under nitrogen. HPLC separation was performed using a silica column. Samples were dissolved in hexane and eluted by a gradient of two mobile phases: *n*-hexane, and *n*-hexane:methanol:2-propanol (40:45:15, by volume). A flow rate of 1.5 mL/min was used with a pressure of 5⁶ Pa, and elution profiles were monitored at 280 nm. Quantification was achieved by spiking serum samples with known concentrations of xenoestrogens before the stripping step, thus determining any inherent loss. For HPLC analysis, calibration curves were produced using xenoestrogen standards. Compounds eluted during the first 10 minutes of HPLC (containing xenoestrogens) were evaporated, re-suspended in ethanol, added to culture medium, and assayed for oestrogenic activity in MCF-7 cells using induction of proliferation and progesterone receptors as endpoints.

STRUCTURE-ACTIVITY RELATIONSHIP MODELLING

Considerable scientific effort has been expended on the development of basic understanding of the processes underlying the interaction of hormonally disruptive chemicals with organisms at the cellular and molecular level. Key aspects of this work are clarification of the interactions that occur between the chemical and the cellular receptor structures, together with the subsequent gene interaction, transcriptional and translational processes. A possible application of such knowledge might be to facilitate the development of predictive mathematical models, based upon structure-activity relationships, that could give early structural alerts of potential hazard during the screening process for novel chemicals. To be of value, such systems would clearly have to address the principal hormonal receptor types. The majority of the available literature to date has focused upon the oestrogen receptor.

As described by Wrenn and Katzenellenbogen (1993), the human oestrogen receptor (hER) is a ligand-regulated transcription factor that mediates the actions of oestrogen in target cells. Upon binding with an appropriate ligand, the hER becomes an active homodimer and enhances transcription at specific gene elements. Deletion analysis of cloned hER cDNA has been used to map the functional domains responsible for DNA and ligand binding, as well as regions involved in dimerisation, nuclear localisation and transcriptional activation. Structure-function analysis of the hormone-binding domain (HBD) has been undertaken to identify residues of importance for the ligand-binding, and transactivational functions of the receptor, and with elucidation of the mechanism by which the receptor differentiates between agonists and antagonists. Despite both oestrogens and anti-oestrogens binding within the HBD, a difference in association must exist to account for the activation of a transcriptional enhancement function when binding is by an oestrogen, whereas anti-oestrogens fully or partially fail in this respect.

Attempts have been made to establish the characteristics necessary for the interaction of a ligand with the oestrogen receptor. As indicated by Jordan *et al.* (1985), the structural requirements are different *in vivo* and *in vitro*. To date, it is believed that the basic structural features required by a compound to produce oestrogenic, anti-oestrogenic or partial oestrogenic action *in vitro* include a strategically located phenolic group on a ring equivalent to an A-ring of the oestran nucleus; this is thought to determine potency. Substitution of the phenol reduces the potency of compounds by reducing affinity for the oestrogen receptor. The correct spacial dimensions are necessary for an oestrogenic ligand to occupy the receptor binding site. The strategically located phenolic hydroxyl (not impaired by alkyl substitutions in the *ortho* position) is necessary for high-affinity binding to the ER and potent oestrogenic activity *in vivo*.

Further structure-relationship studies have attempted to correlate and quantify the chemical attributes which confer the propensity for oestrogenic or anti-oestrogenic potential on a chemical. These have included analysis of a number of compound classes and their substituted derivatives. An early attempt at establishing a model was described by Jordan *et al.* (1985) in which the structural features necessary to initiate or inhibit prolactin synthesis in pituitary cells were studied for oestrogenic and anti-oestrogenic compounds. This approach was again used by Jordan *et al.* (1986) to assess the oestrogenic and anti-oestrogenic actions of a series of triphenylbut-1-enes. The oestrogen triphenylbutene forms the nucleus of the anti-oestrogen tamoxifen, and this has formed the basis for further research into derivatives which may have anti-oestrogenic properties applicable to the treatment of human breast cancer. Jordan *et al.* (1986) commented that precise structure-relationship activity studies are complicated *in vivo* due to the influence of metabolism and that the correlation between, for example, the anti-tumour actions of compounds and their activity in the prolactin synthesis assay is poor. It was suggested that this may possibly be an inappropriate endpoint for the study of structural features necessary to modulate oestrogen-regulated gene

products. If this were the case, it would potentially cast doubt on the validity of any assay based around a tumour cell line and is thus likely to remain a source of controversy.

The use of relative binding affinity of radiolabelled ligands has become a common method of attempting to predict the action of suspected oestrogenic or anti-oestrogenic compounds. The technique is used in conjunction with either libraries of derived compounds or with receptors which have been subject to site-directed mutagenesis. In addition to studies of the effects on prolactin synthesis, Jordan *et al.* (1985) described the results of binding studies using a number of different classes of compounds. This has been built on in more recent work by Anstead and Kym (1995), who studied structure-receptor relationships using benz[a]anthracene diols, and Napolitano *et al.* (1995), who investigated the use of compounds labelled with a carbon-11 isotope. Wrenn and Katzenellenbogen (1993) and Kohno *et al.* (1996) have used mutational analysis of the ligand/hormone-binding domain of the oestrogen receptor to elucidate the stereochemical and structural requirements for recognition and response to ligands. The uses of transfected yeast cell lines are more extensively described in Section 0. In essence, the technique requires the generation of a series of point mutations in the hormone-binding domain of a receptor using site-directed mutagenesis of the ER cDNA. Kohno *et al.* indicated that ligand binding and transactivation are two independent but related processes. This interpretation was based upon the previous demonstration that ligand binding does not appear to affect the binding of ER to its DNA-response element. Ligand binding, however, could possibly alter receptor conformation and influence transactivation. The indication is that the conformation of the receptor is probably altered by binding of specific ligands with differing structures.

The review by Jordan *et al.* (1985) also detailed two assay techniques for use *in vitro* to study prolactin synthesis and progesterone receptor synthesis, in combination with a determination of the relative binding affinity of a compound for the oestrogen receptor.

In these techniques, prolactin synthesis was measured as percentage increase produced by a culture of dispersed cells from immature rat pituitary glands whilst the progesterone receptor was calculated as percentage increase over controls in cultures of dispersed cells from immature rat uteri. The percentage decrease of oestradiol-stimulated progesterone receptors was found from increase in concentrations of antagonist. This review also drew attention to *in vitro* assays avoiding problems of pharmacokinetic confounding factors as encountered with short-acting oestrogens such as oestriol in uterine weight tests. Potencies often appeared low *in vivo* because the steroids were cleared before a biological effect could be produced. This was not the case in sustained release preparations, where high biological activity had been observed. Thus it was considered logical for polyphenolic oestrogens found in the environment to be excreted rapidly following a single daily administration. As a result, the compounds may produce no immediate biological effects *in vivo*, but biological effects can be shown in *in vitro* assay systems, which may better mimic continual exposures of organs to chemicals. The alternative argument can, however, be applied that because *in vitro* models do not simulate metabolic and tissue distribution effects, their predictivity to the in-life situations facing organisms is low. An example is given by the difference between *in vivo* and *in vitro* activity shown for the anti-oestrogen 4-hydroxytamoxifen, which fails to stimulate either progesterone receptor or prolactin synthesis *in vitro*. However, administration *in vivo* results in synthesis of progesterone receptors.

Currently much remains to be learnt about the detailed molecular events that occur during receptor-mediated processes. Additional factors that require clarification include the suggestion by Landel *et al.* (1995) that accessory proteins may be required for optimal interaction of the oestrogen receptor with the oestrogen-responsive elements. The situation has been further complicated by the discovery of possible subtypes for the oestrogen receptor (*e.g.* Pink *et al.*, 1996), which raises questions concerning the

applicability of the cloned receptor currently in use in many of the existing *in vitro* models to the range of potential target tissues *in vivo*.

In view of such outstanding questions, it is apparent that too little is known about the interactions of chemicals with the various cellular receptor types, and the subsequent signal transduction processes, to confidently utilise a mathematically based mechanistic model to routinely and reliably assess, at the cellular level, the hormonal disruptive potential (hazard) of a novel xenobiotic. Whether any such model would be capable of further extrapolation to the *in vivo* situation, and so significantly contribute to risk assessment, is a question which cannot be addressed at the current level of understanding. It is certainly considered worthwhile continuing development of SAR-mathematical models, but it should be accepted that such models are likely to require protracted development and validation before being reliable enough for use as a routine screening tool.

IN VIVO SYSTEMS RELEVANT TO ASSESSMENT OF EFFECTS ON WILDLIFE

INTRODUCTION

A number of non-regulatory *in vivo* test models have been developed which could potentially facilitate the monitoring and risk assessment of environmentally relevant chemicals. Several of these models address non-specific aspects of reproductive development and performance, whilst others have been designed with endpoints indicating specific modulation of the oestrogenic or androgenic hormonal systems.

NON-SPECIFIC

***IN VIVO* FISH STUDIES**

Bresch (1982) test programme

Introduction

Bresch (1982) described a programme of toxicity tests for fish intended to elucidate the long-term action of xenobiotics, with particular reference to reproductive performance. The test programme was designed to detect both inhibitory and stimulatory effects of chemicals on gamete maturation, spawning, fertilisation, embryonic development, hatching and growth. Acute studies were performed on pentachlorophenol, Arochlor, 4-nitrophenol, and a range of metals. Only data for cadmium were presented for the other test protocol.

The zebra fish, *Brachydanio rerio*, was chosen as the animal model by the author because it spawns throughout the year with relatively short cycles and is easy to maintain in aquarium conditions.

Method

For these studies, six-month-old fish were obtained from commercial sources and acclimatised to laboratory conditions for at least two weeks before study.

In order to define the range of concentrations that were likely to encompass a no-effect-level, the author suggested the initial performance of acute toxicity and simple embryo-larval tests:

Acute toxicity:

Adult fish were kept for two days in all-glass aquaria before addition of test material to the medium. Animals were not fed during the study, and three-quarters of the medium was replaced every 24 hours. Five animals were assigned to each of five treatment levels and their survival over a 96 hour exposure period was assessed. The treatment levels differed from each other by a factor of 10^{1/2}. These levels were decided upon on the basis of an initial 48-hour pre-test in which three animals per group were exposed to concentrations differing by one order of magnitude. This test methodology is only suitable for reasonably water-soluble chemicals; flow-thorough systems were recommended for substances of low solubility.

Embryo-larval:

This test used synchronously developed embryos that were placed on study 24 hours after spawning (late gastrula stage). Ten embryos were incubated with 30 mL of medium containing the test chemical in Petri dishes for six days, with replacement of 20 mL of solution every 24 hours. At the end of the exposure period larvae were expected to still contain yolk, thus preventing potentially confounding factors arising from feeding. Animals were immobilised by anaesthetic and their length was measured.

Statistical analysis was evaluated by ANOVA.

Reproduction:

Reproductive tests were performed on seven-month-old fish taken from a batch in which males and females were raised together. They were kept in four groups of 10 females and 17 males in an all-glass, aerated aquarium. Mating generally occurred in the morning, with animals spawning on average at two-day intervals. Eggs were collected in glass vessels covered by a plastic net upon which a spawning substrate of plastic plants was posed. Spawning vessels were replaced when the medium was changed, and eggs counted after removal from the spawning vessel by Pasteur pipette. Viable embryos were identified by their transparency; dead eggs and embryos were recorded on the basis of their opacity. Baseline data were collected over at least 10 spawning cycles and then two groups of fish were exposed for 36 days to test material (in this case, cadmium) at the lowest level. The procedure was then repeated at a 10-times higher concentration with the same animals. At the end of the experiment, 100 embryos were taken from each treated group and incubated in water only. Animals were observed for differences in hatchability and growth over a one-month period. Data were statistically analysed using multiple t-tests.

In a separate experiment, 300 embryos 10 or 20 hours old were incubated in water containing tritiated thymidine. The water was sampled every three minutes over a one-hour period and subjected to scintillation count. The results of this experiment were not reported in the paper.

Endpoints/data interpretation

Acute toxicity:

Approximate LC₅₀: used to establish treatment levels for the embryo-larval test.

Embryo-larval:

Fish length: measured under a microscope. Used to detect lower level toxicity and establish suitable treatment levels for the reproductive assessment.

Reproductive:

Spawning rates: from counting numbers of live and dead embryos at each spawning occasion.

Hatchability and growth: assessed for embryos taken at end of experiment and reared in untreated water.

Considerable variation was reported in the spawning rates over the two-day measurement windows. In the test on cadmium, they detected an apparent reduction in numbers of dead embryos at the low concentration and in numbers of eggs at the high concentration. This effect was such as to necessitate treatment after only 24 hours. The hatchability and growth of embryos taken at the end of the experiment and reared in untreated water were found to be unaffected by treatment.

This test strategy allows study of several spawning cycles within a reasonably short time and could possibly be used as a replacement for a full life cycle test. The total number of eggs was considered to be the best marker, rather than the number of opaque individuals, because the authors considered the total to relate solely to effects on the biochemistry of the female whilst the number of dead also depended upon other actions such as sperm quality and water-related factors.

Wester and Canton (1986) long-term fish test

Introduction

Wester and Canton reported on two long-term exposure experiments on the effects of β -hexachlorocyclohexane (β -HCH) on the Japanese rice fish, Medaka (*Oryzias latipes*).

In this paper, the effects of exposure to the test chemical for up to three months were compared for individuals exposed from the egg stage or from one month after hatching. The study was run as two experiments using the same treatment levels. The levels were based upon a preliminary range-finding study that was not reported.

Method

In the first experiment, groups of fertilised eggs (<24 hour old) were assigned to seven treatment groups (40 eggs per group). Animals were exposed to β -HCH concentrations ranging from 0.032 to 1.0 mg/L. A control group exposed to the vehicle alone was also included. Daily observations were made for mortality or gross changes and, during the embryonic period, heart rate. One and three months after hatching, 10 animals per group were anaesthetised, weighed and their size measured. These animals were then fixed in Bouin's fluid. In addition, for this experiment only, after one month the livers and kidneys were taken from five animals from the control and high dosage groups and placed in glutaraldehyde pending processing for electron microscopy.

The second experiment was similarly structured, except that groups of 25 animals (one month after hatching) were used.

Histopathological examination was performed on the animals fixed in Bouin's fluid. This involved preparation of three serial sections at 10 to 15 levels. The first was stained using haematoxylin and eosin; the remaining two from each position were subject to special staining techniques as necessary, including periodic acid-Schiff, periodic acid-silver methenamine, phosphotungstic acid haematoxylin, Congo-red, Weigert-Mallory van Gieson and Alcian blue. From five animals of the control and high treatment level groups, serial sagittal sections were cut. Six of the most median sections from each fish were selected and stained with periodic acid-Schiff to examine pituitary gland cytomorphology. Glutaraldehyde-fixed tissues were transferred to osmium tetroxide, and ultra-thin sections stained with uranyl acetate and lead citrate were assessed by transmission electron microscopy.

Endpoints/data interpretation

Behaviour/gross abnormality: by visual assessment.

Bodyweight: recorded before humane killing.

Histopathology: by detailed light and electron microscopic examination.

Effects of β -HCH on growth performance and behaviour suggestive of toxicity were reported. The detailed histopathological examination identified effects in several organs including the testes, liver, kidney, thyroid, spleen and adipose tissues. In the case of the testes, this included evidence of intersexuality in some individuals receiving 0.10 mg/L at the three-month stage. At the highest treatment level, adipose tissue was seen to replace a considerable portion of the testis. Effects on the liver included increased vacuolation, cystic lesions and connective tissue at the highest treatment level. On the basis of morphology, evidence of elevation of vitellogenin production was diagnosed. Associated accumulation of vitellogenin was also reported in the renal glomeruli of the majority of the affected fish. The spleen showed an increase in mesenchymal elements. In the thyroid, the size of follicle was reduced and the epithelial cells became cuboidal or columnar in fish treated at high levels whilst the number of PAS-positive cells in the pituitary (considered to be thyroid-stimulating hormone releasing cells) increased.

The authors referred to previous work which they interpreted as indicating that this species does not show spontaneous intersexuality, only undergoing such change in response to xenobiotic interference. On the basis of histopathologically observed intersex change and vitellogenin production, the authors considered

that β -HCH has oestrogenic activity whilst the effects on pituitary and thyroid indicate that this chemical may possess a wider disruptive potential.

Bresch *et al.* (1990) long-term fish test

Introduction

In 1990, Bresch *et al.* published an alternative test strategy, again using zebra fish, that was used to assess the reproductive effects of 4-chloroaniline on fish. The experimental design covered three generations.

Method

Fish forming the F_0 generation were obtained at five months of age from a commercial source and kept under similar conditions to those described above, except that a continuous exchange system was used to provide two complete changes of water per 24-hour period. Other differences included placing spawning vessels in the aquaria in the late afternoons and removing them the following day, approximately three hours after the lights were switched on. Fish were initially acclimatised to these conditions for approximately one week. The spawning capacity of the fish was then assessed over a 10-day period before treatment commenced. Eggs were counted on removal of the spawning vessels and fertilisation was investigated by removing 150 transparent eggs and placing them in Petri dishes. After one day, the number of opaque eggs was counted.

Test material was administered through a pumping system adjusted to maintain the required concentration; flow rates were checked twice weekly and concentrations measured once per week. Three treatment levels were used, with the investigation being run in duplicate. The authors recommended selecting the treatment levels on the basis of LC_{50} and early life stage studies. Egg counts were performed after five, 10 and 17 weeks' exposure. Egg numbers, fertilisation rates and weights were tested for normality using the Shapiro and Wilk method. Mean values for spawning patterns, fertilisation rates and weights were multiply compared using Scheffé's test.

After 21 weeks' exposure, 200 eggs were removed from each group of the F_0 generation and allowed to develop under the continued influence of the test material. Spawning vessels were provided when the fish were 10 weeks old in order to assess sexual maturation. At 19 weeks of age these F_1 animals were weighed. When F_1 fish were 32 weeks of age, samples of 250 eggs were removed from each clutch and reared as for the F_1 generation. These animals, which constituted the F_2 generation, were subject to a similar experimental protocol.

Rearing of the F_1 and F_2 generations was achieved by taking eggs from the spawning vessels and placing them in Petri dishes approximately 2 cm below the water's surface in an aquarium with through-flow of water. Feeding commenced when the larvae started swimming. At six weeks of age, offspring were transferred to large aquaria. Subsequently animals were randomly assigned to spawning aquaria.

Endpoints/data interpretation

Spawning pattern: from counting of eggs.

Fertilisation rates: assessed by counting numbers of opaque eggs after 24 hours' incubation of 150 initially viable eggs.

Sexual maturation: by assessing egg production on provision of spawning vessels to fish of the F_1 generation at 10 weeks of age.

Weight: of F_1 animals at 19 weeks of age.

Morphology: by observation.

In the study of the effects of 4-chloroaniline, spawning pattern and fertilisation rates for the F_0 generation were seen to be unaffected by treatment. Initial development of the F_1 generation was unremarkable; mortality and sexual maturation appeared unaffected by treatment. However, it was found that after five weeks morphological abnormalities were apparent for high-dosage animals, including swollen abdomens and spinal deformities. The weight of animals reared at the low and intermediate concentrations were found to be higher than that of the high treatment level or control groups. Effects were also noted in respect of numbers of eggs produced by the treated animals. F_2 animals displayed similar effects to those of the F_1 generation.

This fish is readily available and easy to breed, and maintenance does not present problems. This model takes a considerable length of time, and a shorter methodology would be preferable but, using examples from this study, it is necessary to expose animals from the time of gamete production by the F_0 generation through to a F_2 generation in order to be able to confidently identify possible delayed effects. It was also pointed out that if the protocol were modified to allow eggs to be taken for the F_2 generation soon after maturation of the F_1 generation, the test could be completed within 30 weeks. Finally, although the test provides little data on spermatogenesis, the authors argued that if fertilisation rates are unaffected it can be assumed that this process is not essentially affected.

Fish short-term alternative studies

Introduction

Landner *et al.* (1985) reported on a short-term laboratory test battery designed to investigate separately a chemical's influence on gamete maturation, spawning, fertilisation, egg hatching and embryo/larval survival. The zebra fish was chosen by these authors on the basis of its ease of adaptability to laboratory conditions, year-round spawning pattern with consistent spawning cycles and times, and rapid rate of development of the young.

Method

Zebra fish were obtained from commercial sources at six to twelve months of age and maintained in aerated aquaria during acclimatisation.

Acute toxicity:

Adult fish in groups of 10 were exposed to one of seven concentrations of the test chemical under semi-static conditions for 96 hours. Medium was replaced at 24-hour intervals.

Embryo/larvae test:

1. Spawning provision: Groups of 120 adult fish were transferred to all-glass aquaria, with replacement of 80% of the water every second day. Over a 10-day period, groups were exposed to media containing the test material or diluent control. Four days before spawning, sub-samples of 10 females and 20 males were selected from each exposure group and held, separated according to sex, in aquaria. Exposure continued throughout this period. On the evening of the fourth day, the sexes from each subgroup were reunited in containers designed to permit spawning. These containers, funnels made of polyvinylchloride, contained a nylon screen that permitted passage of eggs but not fish. In order to promote spawning, each funnel contained a floating nylon "mop". Food was withheld during the spawning period.

2. Offspring: The number of eggs collected from each spawning funnel was recorded each day and non-viable eggs were documented. From the viable eggs collected each day, samples of 50 were held in Petri dishes containing the appropriate treatment medium; between two and four replicates were established. Test solutions were renewed daily, at which time dead animals were counted and removed. Abnormal

larvae were also countered but not removed. Food was not provided. The test continued until all larvae had starved to death, generally within 10 days of hatching.

Opaque egg and hatching success data were assessed by the χ^2 -test. The “Life Tables and Survival Functions” programme of Benedetti *et al.* (1981) was used for hatching time and mean survival time data. The W-test was used for comparing results where more than four replicates were used. Data were analysed by ANOVA.

Larvae:

Additional tests were performed using two-day-old larvae that had previously been untreated. Exposure to a series of test media followed a similar protocol to that for the embryo/larvae test described above, and again continued until death through starvation.

Endpoints/data interpretation

Acute toxicity:

Survival: analysed by the simplified probate method of Litchfield and Wilcoxon and results expressed as a 96-hour LC_{50} with 95% confidence limits.

Embryo/larvae:

The following endpoints were derived from the observations conducted on the offspring:

Percentage opaque (non-viable) eggs.

Hatching success: as a percentage of total eggs that actually hatched.

Medium time from fertilisation to hatching.

Medium time of survival.

Frequency of occurrence of abnormal larvae: as a percentage of total produced.

Larvae:

Median survival time and LC_{50} : were calculated as above.

Both the release of eggs and their viability showed great within-group variations on a daily basis. Once non-viable eggs had been removed, the hatchability of the remainder was, however, high (>95%) and the hatching time was not very variable. For the experiment involving spawning by adult fish exposed to test materials, the parental exposure period before initiating spawning was optimised on the basis of a series of experiments in which a range of effects on the offspring were assessed using varying treatment durations for male and female parents. It was considered that the time taken to spawn might be an additional endpoint. In the fish stock used in the laboratory, naturally occurring deformities only occurred in <0.02% in larvae. Exposure to a series of raw bleach plant effluents did not elicit any increase in the rates of deformity, although they referred to other work that detected effects with chlorinated methoxybenzenes. On the basis of their experience, they did not recommend the use of total number of eggs as an endpoint due to its excessive variability and insensitivity. Level of deformity was considered to be a satisfactory endpoint, at least for the stock used in these tests.

Overall, the authors recommended inclusion of the following tests within the normal environmental hazard assessment battery:

- Pre-exposure of adults for 14 days before spawning to provide offspring for study.
- Comparison of survival (in absence of test chemical) and stress tolerance (in a concentration gradient of the test chemical) of offspring from exposed parents.

- Embryo/larvae tests after direct chemical exposure of eggs and larvae derived from unexposed adults.

SEX DETERMINATION/REVERSAL ASSAYS

Introduction

Evidence has accumulated from both field and laboratory-based studies that the development of sexual morphology in many species is not definitively fixed through genetic mechanisms and may be influenced by a number of environmental factors, including ambient temperature and chemical interference. Variations in such factors may result in development being redirected during early developmental stages or subsequently reversed. This aspect is reviewed by Crews *et al.* (1994), with special reference to the effect of temperature modulation in reptiles. Examples of test systems described as specifically applicable to either oestrogenic or androgenic chemicals are presented in detail in the Sections Sex Determination/Reversal assays, respectively. Two other models are presented in this section. They relate to sex-reversal in chickens, studied by Elbrecht and Smith (1992), and in alligators, studied by Lance and Bogart (1992).

Sex-reversal in chickens

Introduction

Elbrecht and Smith (1992) investigated the action of the aromatase inhibitor (\pm)5-(*p*-cyanophenyl)-5,6,7,8-tetrahydroimidazol [1,5- α]pyridine hydrochloride (AI). Their paper also referred to the study of a range of other chemicals including the aromatase inhibitor 1,4,6-androstatriene-3,17-dione, the non-steroidal inhibitor 6-[(4-chlorophenyl)(1H-1,2,4-triazol-1-yl)methyl]-1-methyl-1H-benzotriazole, testosterone and tamoxifen.

In order to facilitate monitoring of changes in sex expression, an autosexing cross was used. Autosexed chickens are obtained by crossing different breeds possessing sex-linked feather colour mutations, which permits rapid identification of the genotype by feather colour as opposed to phenotypic sexing on basis of cloacal vent shape.

Method

Chicken embryos derived from Rhode Island Red males, *b+b+*, crossed with Plymouth Barred Rock females, *B/w*, were treated with AI on various days of incubation to identify the susceptible window. This was established as days 0 to 7 of incubation. In subsequent experiments, eggs were treated on day 5. Endpoints used were feather colour and cloacal vent structure. In addition, some animals were maintained for up to 31 weeks after hatching before blood samples were collected for analysis of steroid hormones. Gross structure of the gonads was examined and the gonads were removed, fixed in Bouin's fluid, and histopathologically examined.

Endpoints/data interpretation

Feather colour: provided rapid means of sexing on basis of genotype at hatching.

Vent sexing: observation of dimorphism of cloaca used to determine phenotypic sex.

Serum oestradiol and testosterone levels: using commercially available radioimmunoassay kits.

Gonadal histopathology: by light microscopy.

Treatment with AI resulted in nearly all hatchlings showing sex reversal, as assessed by comparison of feather characteristics to cloaca vent shape. Associated changes in gross pathology were observed, whilst histopathology of the sex-reversed females was found to be similar to that for normal males. Measurement of serum hormonal levels showed low oestradiol and high testosterone levels in sex-reversed chickens. Similar sex reversal effects were seen for the other aromatase inhibitor and the non-steroidal inhibitor. In contrast, tamoxifen and testosterone treatment did not initiate sex reversal. On the basis of their findings, the authors considered these effects to be specific to aromatase inhibitors and not amenable to interference by anti-oestrogens or other steroids.

Sex reversal in alligators

Introduction

Lance and Bogart (1992) investigated the action on alligator sex development of three aromatase inhibitors (4-(5,6,7,8-tetrahydroimidazo [1,5 α] pyridin-5yl) benzonitrile monochloride, 4-hydroxyandrostenedione and aminoglutethimide); two anti-oestrogens (tamoxifen and N-n-butyl-11(3,17 β -dihydroxyoestra-1,3,5(10)-trien-7 α -yl)-N-methylundecanamide), an anti-androgen (cyproterone acetate) and two steroids (17 β -oestradiol and dihydrotestosterone). These studies were based upon the effect of incubation temperature on sex in the alligator, with 33°C producing all-male and 30°C producing all female hatchlings.

Method

Alligator eggs were obtained from the wild. After one week's incubation, they were candled and any infertile eggs or dead embryos discarded. Administration of the appropriate test material or vehicle alone was performed on days 7 to 15 or day 17 or 21. This was achieved by drilling a small hole in the shell and aseptically injecting the material into the albumin. The hole was then sealed with paraffin wax and the eggs returned to the incubator at either 33°C or 30°C for 61 and 75 days, respectively. Fetuses were removed from the eggs and killed by decapitation. Gonads, adrenal glands and kidneys were removed, fixed and stained with haematoxylin and eosin or Mallory's stain before microscopic examination by two independent observers under blind-code.

Endpoints/data interpretation

Gonadal histopathology: by light microscopy.

The authors reported a marked increase in mortality between unoperated on eggs and those injected with either vehicle or chemical. This was largely attributed to infections that arose despite the aseptic techniques used. Mortality was also higher for those operated on during days 7 to 15. Evidence of non-gonadal adverse effects on embryos was only recorded for one chemical. A variety of sex reversal effects was also noted. This included complete sex reversal in eggs incubated at 33°C (males) when treated with oestradiol or tamoxifen, and the inhibition of normal ovarian development in some embryos given the aromatase inhibitors. The extreme sensitivity of the embryos to manipulation was noted and, on the available information, the sex determination process and dependencies are not yet fully understood.

OESTROGEN-RELATED

SEX DETERMINATION/REVERSAL ASSAYS

Introduction

An example of a test system developed to investigate the feminisation of animals by environmental oestrogens is the turtle sex determination assay of Bergeron *et al.*, (1994) which is a bioassay system employing the red-eared slider turtle, *Trachemys scripta*.

Trachemys scripta is a species that possesses a temperature-dependent sex determination pattern in which warm incubation temperatures (*e.g.* 31°C) produce all-female hatchlings and cooler temperatures (*e.g.* 26°C) result in all-male hatchlings. Intermediate temperatures result in varying sex ratios.

Method

In the study, eggs were randomly assigned to treatment groups, 15 eggs per group. The eggs were spotted with either varying (environmentally relevant) doses of polychlorinated biphenyls (PCBs) in ethanol, a mixture of PCBs in this vehicle, a positive control (17 β -oestradiol) or vehicle alone. The study commenced with eggs approximately four weeks after laying (equivalent to the beginning of gonadal development). The eggs were incubated on a layer of vermiculite:water (1:1) and incubated at controlled temperatures (27.8 or 26°C) until hatching (after approximately seven weeks). Monitoring of embryonic development was initially by candling eggs and later by dissecting a small subsample of animals at approximately twice-weekly intervals in order to determine specific developmental stages. Hatchlings were dissected to determine sex ratios for the groups. Gonadal sex and genital duct status were noted. Results were subject to statistical analysis using the Fisher's Exact test.

Endpoints/data interpretation

Gonadal sex and genital duct status: determined by visualisation under a dissection microscope. Sex was subsequently confirmed by histopathological examination.

Significant feminisation was recorded for two of the congeners tested, 2',4',6'-trichloro-4-biphenyl and 2',3',4',5'-tetrachloro-4-biphenylol, with synergistic activity being observed when they were applied in combination. In all cases in which the gonad was female, oviducts or Müllerian ducts were present. Where testes were detected in treated animals, Müllerian ducts were apparent.

GOLDFISH REPRODUCTIVE HORMONE ASSAY

Introduction

The widely used vitellogenin induction assays are discussed above. Other biochemical or hormonal markers for oestrogenic exposure might be possible in the intact animal. As an example, the work of MacLatcy and van der Kraak (1995) on reproductive hormone levels in goldfish in response to administration of the phytoestrogen β -Sitosterol is discussed below.

Method

This paper presents a series of experiments investigating effects of intraperitoneal injection of a phytoestrogen on goldfish, possibly with subsequent administration of Ovaprim, followed by measurement of plasma sex hormones and *in vitro* gonadal steroid hormone production. The basic methodology was as follows:

Common or comet varieties of goldfish were acclimatised to the experimental conditions for one week. On days 0 and 2 of study, the fish were anaesthetised and injected with the appropriate dosage of test material in vehicle or vehicle alone (sesame oil). On day 4 they were again anaesthetised and bled by caudal puncture. Plasma testosterone (T) and 11-ketotestosterone (11-KT) levels, in males, and T and 17 β -oestradiol (E₂) plasma levels, in females, were measured. In some cases plasma gonadotrophin II (GtH-II) level was also analysed. In one experiment gonads were removed, sections incubated in an appropriate medium and *in vitro* gonadal pregnenolone and T levels. Group sizes were eight to nine fish of each sex per group for the plasma investigations and three to four per group per sex for the *in vitro* experiment. Statistical analysis of plasma hormonal levels used Duncan's Multiple Range test whilst gonadal steroid production levels were averaged, the pool for each fish being used to derive a group mean. One-way ANOVA was then applied.

Endpoints/data interpretation

Plasma T, 11-KT and GtH-II levels: measured by radioimmunoassay.

Plasma E₂ level: measured by the method of van der Kraak, 1990.

Gonadal T and pregnenolone levels: measured by radioimmunoassay.

These studies on the goldfish demonstrated that β -Sitosterol treatment resulted in lowered plasma T and 11-KT levels in males and T and E₂ levels in females, thus indicating a similar mode of action of the test material in both sexes. There was evidence of dosage-related response in each case. The changes in plasma hormones paralleled the actions of E₂ on the reproductive performance of goldfish. Associated changes in production of hormones from the gonads were also observed, providing confidence in the validity of the plasma level changes.

INVERTEBRATE ASSAYS

Introduction

On the basis of reports for various invertebrate species of the presence of endogenous oestrogens and aromatase activity, and of a variety of reproductive effects following oestrogen administration, Baldwin *et al.* (1995) investigated potential physiological and biochemical target sites for oestrogenic xenobiotics in the crustacean, *Daphnia magna*. The effect of diethylstilboestrol (DES) on daphnids was studied following acute exposure and over two generations.

Method

Daphnids were maintained using routine culture techniques, 40 to a beaker. Animals were fed on unicellular green algae.

Acute exposure: Neonatal (<24 hours) or adult (12 \pm 1 days) daphnids were exposed to various concentrations of DES for 48 hours. Survival, moulting frequency and growth were assessed for the neonatal animals. LC₅₀ values were calculated by binomial probability. Adults were assessed for steroid metabolism following the exposure period.

Chronic exposure: DES or vehicle alone was added at the appropriate concentration to test beakers; 10 beakers were used per test level. One daphnid (<24 hours old) was added to each beaker. Test medium was replaced three times each week and the animals were observed daily for survival, moulting and reproduction. After three weeks the first-generation animals were assayed for steroid metabolic capability. A second generation was similarly exposed using neonatal offspring, and at the end of the study the length of the second-generation animals was determined.

Endpoints/data interpretation

Length of second-generation animals: determined microscopically.

Survival, moulting frequency and growth: by observation.

Steroid metabolism: testosterone metabolism assessed by exposing groups of five daphnids (still exposed to DES) to [¹⁴C]testosterone for 16 hours. The radiolabelled metabolites of testosterone were then extracted, separated by thin-layer chromatography, identified using authentic standards, and quantified by liquid scintillation spectrometry. Soluble protein levels were determined using the Bradford method.

The acute test showed that immature but not adult daphnids were sensitive to the effects of DES on moulting. Clear evidence of an effect on steroid metabolism was not derived from the multigeneration study (see below) due to a high level of within-treatment variability. Clarification was sought from the acute exposure study by investigating glucose and sulfate conjugation of testosterone; effects on glucose but not sulfate conjugation were established. From these results it was suggested that steroid metabolic capacity, if measured during acute exposure, may be predictive of toxicity by such chemicals.

The multigeneration study demonstrated effects of DES on frequency of moulting in first-generation juvenile daphnids. First-generation adults and the second generation were not affected in this respect, indicating possible acclimatisation to this effect of treatment. The second-generation daphnids were also not affected in respect of total length. A clear reduction in the number of offspring was, however, shown for these second-generation adults; this was attributable to a reduction in the average brood size.

ANDROGEN-RELATED

SEX DETERMINATION/REVERSAL ASSAYS

Introduction

As detailed in the Section Sex determination/reversal assays, sex reversal may occur as a response to chemical exposure in several species. Systems for the investigation of the masculinisation effects of androgenic chemicals are presented below.

Female mosquitofish responses

Introduction

Following previous reports of populations of sexually dimorphic poeciliid mosquitofish (*Gambusia affinis holbrooki*) associated with paper mill effluent exposure, Denton *et al.* (1985) developed a laboratory model using this species to investigate the potential androgenic activity of soya bean extracts.

Method

The test materials used for this study were commercial extracts of soya bean containing a preponderance of either β -sitosterol or stigmasterol. *Mycobacterium smegmatis* in a nutrient broth was added to the extracts degrade the plant sterols. It was considered that the mycobacterium was likely to produce androstene-like chemicals.

Locally caught female fish were kept in holding tanks until use on study. For the study, groups of three were housed in 2 L beakers (60 animals in all). A pierced cellophane bag was added to each beaker. This either contained the plant extract with mycobacterium culture or was empty. The animals were observed during the exposure period.

Endpoints/data interpretation

Physical morphology: by visual examination.

All fish exposed to the plant extract developed male-like gonopodia. In some cases changes were apparent within six days, with further masculinisation over a two-week period: these characteristics did not show reversal when exposure to plant products was withdrawn.

Female tadpole responses

Introduction

Yu *et al.* (1993) demonstrated that female tadpoles of *Rana catesbeiana* showed changes characteristic of sex reversal following intraperitoneal implantation of 4-hydroxyandrostenedione (4-OHA).

Method

Over-winter tadpoles of *Rana catesbeiana* were maintained in dechlorinated tap water, at a temperature of $20 \pm 1^{\circ}\text{C}$, in order to prevent heat-associated sex reversal. When at metamorphic stages X to XII (approximately nine months old), the tadpoles were laparotomised and females selected for study. A silastic capsule was implanted onto the mesentery of each animal. The capsule was either empty (control) or contained crystalline 4-OHA (treated group). After three months, animals were humanely killed and the gonads of a proportion of animals were taken into Bouin's solution for subsequent paraffin embedding, haematoxylin staining, and histopathological examination. The gonads from other tadpoles were taken, sectioned into small pieces and pooled. Tubes each containing 30 mg of gonadal tissue were incubated using a suitable medium for six hours. The supernatant from the medium was subsequently assayed for oestradiol (E_2) and testosterone (T).

Endpoints/data interpretation

Pathological assessment: assessment of gross and histological appearance of gonads.

Gonado: somatic index (GSI): calculated as gonad weight divided by bodyweight, expressed as a percentage.

Gonadal hormonal level: E_2 and T measured using proprietary radioimmunoassay kits.

Treatment with 4-OHA resulted in a masculinisation of the gonads; this was in the form of a change in gonadal appearance and reduction in size and weight, with increasing degree of masculinisation. Histologically affected gonads could be categorised into degenerating ovaries, intersexes and testes. The reduction in gonadal size with androgen treatment was reflected in changing GSI values. Hormonal assay showed a reduction in gonadal E_2 levels and increased T level in response to treatment.

IMPOSEX IN MOLLUSCS

Introduction

Following the widespread detection of sexual malformations (imposex) in wild populations of molluscs, a number of laboratory models have been developed to study the phenomena. The term "imposex" was used to describe development of male primary sexual characteristics in female gastropods. This may range from the development of a penis close to and behind the right tentacle (the normal position in the male) to the development of a superficial vas deferens between the genital papilla and the penis, in the more extreme cases. These structures may even overgrow and occlude the papilla, preventing egg liberation and breeding. Laboratory experiments have now firmly established organotin chemicals (such as tributyl tin, TBT) as the causative agents.

Method

Bryan *et al.* (1986) demonstrated the imposex effect of TBT in dogwhelks using tidal tank experiments in which TBT was leached into the water.

Subsequent development

Further tidal tank experiments by **Smith and McVeagh (1991)** examined the incidence of imposex in dogwhelks at approximate concentrations of 0.01, 0.1 and 1.0 µg/L.

An experiment by **Gibbs *et al.* (1988)** examined the degree of imposex in dogwhelks over a two-year period from the time of hatching, when exposed to various concentrations of TBT leachate from antifouling paints. TBT concentrations used were 1-2, 3-5, 20 and 100 ng tin/L.

Further work by **Bryan *et al.* (1988)** investigated the capacity of various organotin compounds to induce imposex in the dogwhelk. Animals already slightly affected by imposex were taken from the wild and exposed to 200 ng/L tin in the form of TBT chloride, tri-n-propyltin (TPrT), tetrabutyltin (TTBT), dibutyltin (DBT) or triphenyltin (TPhT) for 14 days before being returned to the shore.

In an attempt to identify which part of the neuroendocrine system of the marine gastropod, *Ocenebra erinacea*, was associated with the induction of imposex, **F□ral and Le Gall (1983)** used isolated female pedal ganglia, nervous systems and presumptive penis-forming areas in a biological assay.

Oehlmann *et al.* (1996) have also investigated steroid levels in response to TBT in *Nucells lapillus* and *Hinia reticulata*.

Endpoints/data interpretation

Relative penis size (RPS): to allow for different average sizes of molluscs in different populations and differences in population penis sizes, penis size is calculated by the formula:

$$(\text{mean female penis length})^3 / (\text{mean male penis length})^3 \times 100$$

The length of the penis is usually measured from its tip to the junction with the bodywall, but some differences in procedure have occurred. Bryan *et al.* (1986) used a 1 mm piece of graph paper to measure the penis underwater and made no attempt to straighten the penis. Other studies have used callipers, optical micrometers, stereomicroscopes or a graticule under a low power binocular microscope (Short *et al.*, 1989; Ellis and Pattisina, 1990; Smith and McVeagh, 1991). The vas deferens sequence index has been used as a marker for imposex, but RPS is considered the more practical index because it is easier to measure penis size than vas deferens conditions (Short *et al.*, 1989).

Bryan *et al.* (1986) stated that after four months the dogwhelks accumulated 1 mg/g of tin in the tributyl fraction and achieved a 40% degree of imposex, which was still increasing. Dogwhelks transplanted to Plymouth Marina gave comparative results.

Smith and McVeagh saw a high mortality rate (52%) at the highest concentration with 92% of the surviving females developing a penis. At the intermediate concentration, overall mortality was 57% and 50% of the females developed a penis, whilst for the low concentration mortality was only 6% but imposex occurred in 95% of the females.

Gibbs *et al.* (1988) reported that all exposed females developed a penis, with sizes of between 50-60% of the male penis bulk being recorded after TBT exposure for one and two years, respectively, at 1-2 ng

tin/L. On exposure to TBT levels of 3-5 ng tin/L, the female penis was similar in size to that of the male. Increasing TBT concentrations led to further male characteristics developing and repression of female characteristics. By 1-2 ng/L some females were sterile as a result of oviduct blockage whilst at 3-5 ng/L virtually all females were sterile but still showed apparently normal oogenesis. Oogenesis was suppressed, oocytes reabsorbed and spermatogenesis initiated at 10 ng/L. At 20 ng/L, a functional testis had formed in the females.

Bryan *et al.* (1988) showed female penis size (as a percentage of male penis size) to be 44% in those exposed to TBT chloride compared to 6% in the controls. Values for females exposed to TPrT increased to 14%; other compounds had no effect. It was suggested that the imposex effect was not specific to tributyltin, as TPrT also increased the incidence of imposex.

F□ral and Le Gall showed that culturing of pedal ganglia with the presumptive penis-forming area did not produce a penis, nor did culturing of the complete nervous system in clean sea water. However, culturing of whole nervous systems in polluted sea water caused penis development. Culturing in artificial sea water with added TBT (0.2 µg/L) also caused penis growth. The authors concluded that TBT primarily affects the cerebropleural ganglia of the snails.

Oehlmann *et al.* (1996) showed increased levels of testosterone associated with the advancing stages of imposex. Another experiment demonstrated that testosterone titres in the tissue increased with increasing TBT concentrations and duration of exposure. Testosterone, in the absence of TBT, also induced imposex. The role of testosterone in induction of imposex was further clarified by simultaneously exposing dogwhelks to high concentrations of TBT and to a competitive inhibitor of androgen receptors, cyproterone acetate. Imposex was completely suppressed under these conditions. If TBT inhibits aromatisation of androgens, a specific steroidal aromatase inhibitor would also be expected to induce imposex. Indeed, when the steroidal aromatase inhibitor SH 489 (1-Methyl-1,4-androstadien-3,17-dion) was compared to a solvent control and a TBT exposure group with 5 ng TBT as Sn/L, imposex was found to be significantly greater than that in the controls. It was concluded that imposex appears to be due to TBT-induced inhibition of the cytochrome P-450-dependent aromatase.

IN VITRO SYSTEMS RELEVANT TO ASSESSMENT OF EFFECTS ON WILDLIFE

INTRODUCTION

Although many *in vitro* test models have been developed to assist in the assessment of the potential sex hormone-disruptive capacity of chemicals, most are of potential use in assessing the risk to humans are reviewed in the Section *In vivo* systems relevant to assessment of toxicity to humans. A few systems exist, however, that are wildlife rather than animal focused; these are reviewed below.

OESTROGEN-RELATED

HORMONAL/RECEPTOR-BINDING ASSAYS

Introduction

In addition to the *in vitro* assay for vitellogenin induction discussed above, a number of test models have been developed which measure the potential hormonally disruptive capability of chemicals in terms of receptor interactions.

Thomas and Smith (1993) presented an alternative screening assay for oestrogenic activity that measured the levels of oestrogen receptor (ER) binding in the liver of the spotted sea trout (*Cynoscion nebulosus*).

Ho et al. (1988) published an account of the development of reliable methodology to measure cytoplasmic and nuclear ER binding in liver from the turtle, *Chrysemys picta*.

Method

Thomas and Smith: The livers of eight adult spotted sea trout were taken and deep-frozen. Before study, livers were thawed, homogenised, centrifuged at 104,000 g for 90 minutes, and the cytosolic extracts pooled. Cytosolic extracts were incubated with tritiated oestradiol alone at 2 nM or in the presence of competing ligands at a range of concentrations (0.01-1 nM) for 24 hours at 4°C. Dextran-coated charcoal was then added to separate free from bound radiolabelled oestradiol. Assays were performed in triplicate.

Ho et al.: Sexually mature turtles were obtained from commercial sources and the animals killed by decapitation. The plastron was removed to allow access to the left and right liver lobes. These were taken into ice-cold buffer solution and all subsequent tissue fractionation processes were performed in the presence of a protease inhibitor at this temperature. Liver was homogenised and centrifuged at 1000 g for 20 minutes. The supernatant was further centrifuged at 105,000 g for one hour to give the cytosolic sample. The pellet from the low-speed centrifugation was re-suspended, filtered, and the filtrate centrifuged at 800 g for 10 minutes to obtain the nuclei. Nuclear extract was obtained by re-suspension and high-speed centrifugation of the nuclei. Aliquots of both the nuclear and cytosolic samples were deep-frozen pending subsequent analysis (within a two-month period).

The oestrogen receptor content of the cytosolic and nuclear extracts was determined by one or more of the following procedures:

Dextran-coated charcoal assay: Aliquots of the appropriate extract were diluted in buffer and incubated with [³H]oestradiol in the presence or absence of 100-fold diethylstilboestrol. Free or loosely bound steroids were removed by treatment with dextran-coated charcoal and Dextran T-70. Following centrifugation, radioactivity of the supernatant was measured using scintillation spectrophotometry and specific oestradiol binding was calculated.

Hydroxylapatite (HAP) assay: Aliquots of the appropriate extract were diluted in buffer and incubated with [³H]oestradiol in the presence or absence of 100-fold diethylstilboestrol. Subsequently, an equal amount of HAP slurry was added and the assay tubes vortexed and incubated. After centrifugation, the supernatant was discarded and the HAP pellet repeatedly washed. Protein-bound steroid was then extracted using ethanol and radioactivity was again measured using scintillation spectrophotometry.

DNA-cellulose column chromatography: Chromatography was also attempted for the cytosolic extract using single-stranded calf thymus DNA-cellulose.

Endpoints/data interpretation

Thomas and Smith (1993):

Percentage binding of [³H]oestradiol: calculated from measurement of free and bound radiolabelled oestradiol and expressed as the concentration required to depress specific [³H]oestradiol binding by 50%.

Diethylstilboestrol and oestradiol showed similar levels for competition with the ER. The anti-oestrogens clomiphenine, tamoxifen and nafoxidine also bound to the receptor, but were markedly less potent than oestradiol in contrast to the pesticide Kepone, which had a significantly greater potency than oestradiol. Significant interactions were also observed for copper, zinc, cadmium and selenium oxide ions. A number of other xenobiotics that are oestrogenic in mammals did not bind with sea trout ER; these included DDT and PCB mixtures. This was considered to indicate species differences in receptor specificity that would be expected to result in difficulties in extrapolating between fish and mammals.

Ho *et al.* (1988):

Oestrogen receptor concentration: measured for cytosol and nuclear extracts using dextran-coated charcoal, HAP or DNA-cellulose column chromatography assays.

Specific oestrogen binding: calculated by subtraction of radioactivity measured in the presence of unlabelled steroid (non-specific binding) from that measured in the absence of unlabelled steroid (total binding).

For estimation of ER binding, the dextran-coated charcoal assay procedure had a number of drawbacks, including

- 1) the non-displaceable portion of the bound radioactivity usually constituting more than 65-70% of the total bound radioactivity in the cytosol;
- 2) duplicate assays of a sample often varying by 20-30%, whereas interassay variations were 25-35%; and
- 3) the dextran-coated charcoal only displacing a portion of the oestradiol-displaceable counts, whilst dihydroxytestosterone and progesterone were all partially effective in competing for the radiolabelled binding sites.

The HAP procedure showed only 55 variations on duplicate samples, and non-specific binding was less than 30% of the total bound radioactivity. Interassay variation was only 5-15%. The DNA cellulose column chromatography showed non-specific binding usually below this level; however, when compared to the HAP procedure, levels of high affinity binding were very low. On the basis of the data, the authors recommended the HAP procedure for measurement of cytoplasmic ER binding.

In contrast, in the case of the nuclear ER binding, the non-specific binding was low for both the dextran-coated charcoal and HAP assay methods (less than 30% of total bound). The HAP procedure was found to measure only 30-40% of the binding found by the other method, and hence the dextran-based method was preferred for processing of the nuclear extracts.

ANDROGEN-RELATED

ANDROGEN FORMATION IN CARP

Introduction

Yu and Lin (1986) reported on an *in vitro* assay that measured androgen formation by carp (*Cyprinus carpio*) testes principally as a bioassay for piscine gonadotrophins, to assist in the identification of such chemicals and to allow comparison with gonadotrophins from various vertebrate species.

Method

Mature male carp were obtained commercially, humanely killed by decapitation and the testes collected; four to six testes were pooled for each incubation. Several methods of preparation of the testes were investigated:

- 1). **Minced:** Testes were cut into approximately 1 g blocks, immersed in Hank's balanced salts solution, chopped by use of a blender and, after appropriate mixing and washing, the tissue was allowed to sediment and subsequently maintained in a suitable culture medium. Aliquots of the testis suspension were incubated for four hours at 25°C in 20 mL glass vials with a gonadotrophin derived from silver carp and carp. Supernatant was then collected and deep-frozen pending assay for androgen activity.
- 2). **Slices:** Testes were sliced into pieces weighing approximately 25 mg, and four such pieces were pre-incubated and added to vials using similar procedures to 1). At the end of the incubation period, supernatant was collected and deep-frozen pending assay for androgen activity.
- 3). **Minced/collagenase treated:** Minced testes prepared as for 1), were further treated by use of a collagenase dispersion, the suspension filtered, and androgen-forming activities of suspension, filtrate and residue compared.
- 4). **Homogenised testis:** 5 g samples of testes were mechanically homogenised in a suitable medium, then pre-incubated and exposed to gonadotrophins, as for 1).

Endpoints/data interpretation

Androgen concentration: determined by radioimmunoassay involving incubation of the medium with tritiated testosterone and anti-testosterone serum for 60 minutes, separation of unbound steroids from bound forms using dextran-coated charcoal, and counting by liquid scintillation spectroscopy. Total androgen concentration was measured; sensitivity was 5 pg testosterone per tube.

Large amounts of androgens were found using testes slices or minced testes when stimulated with gonadotrophin; dosage relationship was observed. The use of mincing and collagenase dispersion was similar in effect to just mincing in activity with the majority of the androgen being found in the residue fraction following filtration. However, a considerable loss of total androgen level was found under these conditions. Concentration of androgen was very low in the homogenised samples. Although levels were highest in the slices, the minced testis was selected for routine use because it was considered to be more homogeneous given that there were differences in the rate of the development and maturation of different parts of the carp testis. The homogenate was also considered to be more convenient and gave consistent preparations.

The model system was used to show that there was *de novo* synthesis of androgen, not just secretion of the material in response to stimulation. This was achieved by measuring the levels of androgen in the testis and incubation medium before and after stimulation with gonadotrophin. The effects of gonadotrophin stimulation were found to be enhanced by co-administration of theophylline but not of xanthine.

ANNEX

EXECUTIVE SUMMARY OF EMWAT REPORT (Tattersfield *et al.*, 1997)

Scope and Objectives

The EMWAT workshop brought together about 50 leading experts from fields of direct relevance to endocrine modulating effects. The primary purpose of EMWAT was to address the issue of hazard identification of endocrine modulators (EMs) in wildlife.

The *scope* of the workshop covered:

- hazard identification of endocrine modulators;
- terrestrial and aquatic wildlife;
- all chemicals, including general industrial substances, pesticides and pharmaceuticals;
- contaminated environmental media.

The workshop *objectives* were to:

- assess the need for tests and procedures which can identify endocrine modulators, both at the stage of product development, and in effluents and environmental media;
- critically evaluate the available test methods in terms of their ability to identify endocrine modulators, and to propose modifications, as appropriate;
- identify gaps in the existing suite of test methods, and to propose new methods to fill those gaps;
- recommend appropriate hazard identification strategies applicable to endocrine modulators in wildlife.

The workshop was divided into a series of plenary sessions and working groups covering the following five areas:

- Structure Activity Relationships (SARs);
- *In vitro* tests;
- *In vivo* tests;
- Field survey techniques, including toxicity identification and evaluation (TIE);
- Hazard identification strategies.

The members of each of the working groups are listed at the beginning of each chapter and all workshop participants are listed in Annex 1.

Conclusions and recommendations

The output of the EMWAT workshop is the result of a balanced attempt to determine the feasibility of identifying the hazard of EMs to wildlife, based on current knowledge. A realistic approach for the development of testing methodologies, and a strategy for identification of the hazard associated with EMs, is proposed. There is a recognition that there are substantial gaps in knowledge of endocrine modulation in wildlife such that further research is required before the most appropriate ways to address some aspects of this issue can be determined.

Full reports of the discussions, conclusions and recommendations on SARs, *in vitro* tests, *in vivo* tests, field survey techniques and hazard identification strategies are given in Chapters 2, 3, 4, 5 and 6 respectively. The major conclusions and recommendations are summarised below.

Structure Activity Relationships (SARs)

There is some optimism that SARs will be available in a relatively short time frame (3 years), at least for a limited number of receptors and restricted classes of compounds. However, this view may need to be balanced by experience of the use of SARs for identification of potential carcinogens. In the case of carcinogens, initial expectations of the use of SARs proved overly optimistic (Ashby, 1994). Development of SARs should continue given the potential to assess large numbers of compounds and their potential usefulness for alerting attention to structural features which indicate potential ability to have intrinsic activity. SARs will not, however, be predictors of reproductive or developmental effects in wildlife. It would be reasonable, with current knowledge, for this initial development to proceed with a focus on predicting interactions with estrogen and androgen receptors. The main conclusions concerning the development of SARs in this field are listed below:

- SARs to predict binding affinities of the arylhydrocarbon receptor, the estrogen receptor and the androgen receptor should be available within the next three years, assuming adequate research support. Development of models for binding affinity to the thyroid receptor may also be possible in this time-frame.
- Models appropriate for other mechanisms that might result, for example, in estrogenic effects *in vivo* (e.g., changes in ligand synthesis), will not be available in that time-frame but the development of SARs for other additional mechanisms of action should continue.
- An important aspect of the delay in developing SARs descriptive of mechanisms other than receptor binding is the current lack of assay systems (e.g., *in vitro*) needed for developing the databases to derive robust SAR models.
- High quality SARs should be used as soon as they are available, i.e. it would be inappropriate to delay use of SARs until all the mechanisms can be modelled.
- Collaboration in model development should be undertaken to avoid duplication and to stimulate the integration of different approaches. This will result in sets of models instead of a single model for endpoints of interest.
- Proposed models should be evaluated during development by using training and validation data sets and/or cross validation analyses. Models should be formulated in a transparent manner and must be clearly documented.
- Models should be evaluated in an international forum by a panel of experts in the areas of SAR development and ecological risk assessment. Results of evaluations should include recommendations on the strengths and weaknesses of using different models singly or in suites.

In vitro Testing

In vitro methods are very useful for assessing the intrinsic activity of large numbers of compounds and elucidating mechanisms of action subsequent to the determination of reproductive or developmental effects. Before *in vitro* tests can be used routinely, suitable tests must be selected, they must then be optimised and validated using internationally agreed approaches. The science and methodology requires substantial development before *in vitro* tests may be used routinely as part of the hazard identification of EMs. In addition, as a matter of urgency, the issue is currently being dealt with at higher policy levels within OECD. Information from *in vitro* tests must be verified *in vivo* as, at present, *in vitro* tests alone are not capable of predicting effects on reproduction or development.

- *In vitro* test methods have utility for elucidating mechanisms of action, and could be used in conjunction with SAR to prioritise compounds for short term *in vivo* testing.
- Mechanistic understanding will be an important factor for identifying endocrine modulators and must be used together with knowledge on comparative endocrinology in order to extrapolate potential effects in different wildlife species.
- *In vitro* tests are complementary to, but not substitutes for *in vivo* tests. Hence activity in an *in vitro* assay requires verification *in vivo*.
- *In vitro* assays are useful for detecting chemicals which act through direct, receptor mediated mechanisms of action, and there is the potential to further develop systems to detect compounds acting through alternate mechanisms of action.
- The utility of *in vitro* methods is limited by a number of important disadvantages, including lack of metabolic activity and lack of predictiveness for *in vivo* effects.
- It is difficult to explain the process which would cause synergy in cell-free systems and in cell systems inducible via a single mechanism of action. It was concluded that there were fundamental problems associated with interpreting synergy *in vitro*.
- Our lack of knowledge regarding the extent and significance of species differences (e.g. in differences in steroid receptors/steroidogenic enzymes) is a concern, since this understanding is needed to provide a scientific database to justify species to species predictions.
- At present there are no nationally or internationally harmonised *in vitro* test methods for wildlife. The following approach to initiate this process is recommended:
 1. identification of two of the most promising assays, assessing two different modes of action (one assay to assess effects on steroidogenesis and one assay to assess receptor-binding are proposed initially);
 2. validation using agreed approaches (ECVAM/ICCVAM);
 3. further development and validation of additional assays.

***In vivo* Testing**

In vivo techniques, at this stage, are the only methods capable of determining effects of endocrine modulators on reproduction and development in wildlife. As such they are essential at both prioritisation/screening and confirmatory tiers of testing for EMs. Many current *in vivo* testing methods used for regulatory purposes include reproductive and developmental endpoints and may be optimised for detection of EMs. Modifications of existing test methods must be fully validated and an understanding of the implications of often subtle effects observed in some *in vivo* tests must be gained. Problems with reproducibility of these often subtle endocrine modulation effects are already being encountered (Ashby and Elliott, 1997). It is important to recognise that confounding factors may influence results of such tests, for example, minor changes in some reproductive parameters have been shown to be affected by small changes in body weight resulting in difficulties in the interpretation of results (compare Sharpe *et al.*, 1995, with Ashby *et al.*, 1997).

Comparative endocrinology and species differences must be considered in relation to the diversity of wildlife species we seek to protect. It may be that at the molecular level (e.g. as examined in *in vitro* testing) there is a sufficient degree of conservation to allow some reasonable species to species extrapolations. The ability to extrapolate results of *in vivo* and *in vitro* tests on a limited number of species to other wildlife species, requires an understanding of the degree to which species differences are important. Therefore the scientific database on this aspect must be further developed in order to justify such species to species predictions.

- *In vivo* screening and testing systems for EMs have advantages over *in vitro* systems in that they are highly integrative, facilitate the evaluation of mixed mechanisms of action and can be used to evaluate numerous endpoints (from molecular markers to gonadal histology).
- A tiered *in vivo* testing strategy is recommended involving screening/prioritisation at tier 1, subchronic tests at tier 2 and chronic testing at tier 3 (see Table 1).
- *In vivo* tests can be supplemented by the use of *in vitro* procedures in order to elucidate the mechanisms of action, and SARs would also be of assistance at the tier 1 screening stage.
- Although no currently available and validated *in vivo* tests are appropriate, as they stand, for detection of EMs, several could be readily adapted and others might form the basis of useful tests after further development.
- Detailed recommendations were made for tests with mammals, birds and fish.
- No recommendations were made for the development of tests with reptiles and amphibians. Rather, it was proposed that case-by-case investigations could be made when effects on these organisms are observed in the field.
- Invertebrates were identified as an important group of organisms for which test methods need to be developed. However, not enough is known of the effects of toxicants on invertebrate endocrine function to design predictive test methods, nor is there sufficient confidence that the kinds of endocrine modulation observed in vertebrates (such as estrogenic effects) can be easily extrapolated to invertebrate systems.
- To ensure continued improvement, it is recommended that a peer review process be utilised to review the *in vivo* screening and testing methods adopted. At a given time after implementation, the review process should assess the utility of the information generated and which of the methodological modifications have proven most useful.

Field Surveys and Toxicity Identification and Evaluation (TIE)

- Field surveys and TIE approaches are considered to play an important role in detecting endocrine modulating effects in wildlife, and programmes to determine if there are general effects by yet unknown chemicals are needed.
- A strategy for an eco-epidemiological approach to the detection of endocrine modulating substances was proposed:
 - ⇒ Phase I: Generalised Screening surveys: screening at the population level to determine the general status of wildlife populations in a locale (e.g. using broad range of indicators at the community, population and individual level)
 - ⇒ Phase II: Identification of causality via a combination of analytical determinations and verifications of effects: (e.g. using combination of chemical analysis, selective/stratified sampling, TIE, bioassay-directed fractionation)
 - ⇒ Phase III: Surveying directed at specifically looking for endocrine-mediated effects: assessment of the magnitude and extent of effects and the risk relative to other stressors
 - ⇒ Phase IV: Validation of field observations (e.g. using controlled laboratory studies, *in situ* bioassays with whole organisms)

Note that the phases are not necessarily sequential and one might move up and down the scheme depending upon the aims of the study.
- Point sources such as effluent outfalls, incinerators and treated fields should receive the highest priority for monitoring.
- Co-ordinated monitoring programmes which cross international boundaries and incorporate the expertise of scientists from different disciplines will maximise the possibility of finding evidence of endocrine disruption in wildlife including in areas which are not necessarily heavily contaminated.

Hazard Identification Strategy

A scheme is proposed for the hazard identification of EMs particularly focusing on ***chemicals regulation*** although it could be adapted to apply to effluents. It should be borne in mind that chemicals legislation is only one approach to lower the risk associated with environmental pollution and is complementary to the eco-epidemiological approach as applied through field surveys. The hazard identification scheme proposed is a tiered process but the triggers for moving between the tiers need to be developed and internationally agreed. At the prioritisation/screening tier, short term *in vivo* tests are essential and may be supplemented by *in vitro* and SAR information. However, at present, there are no appropriate short term *in vivo* tests for EMs available so these *in vivo* tests need to be developed and validated as a priority.

- A proposal for a hazard identification (testing) strategy involving three stages was developed:
 - ⇒ Initial Assessment - using available information to determine whether there is evidence that a particular substance may cause endocrine modulation (e.g. SAR predictions, data on toxicity, fate, production and use of the chemical, evidence from mammalian studies for human health, field monitoring, etc.) An aid to this initial assessment (the Grey Scale) is suggested.
 - ⇒ Screening/Prioritisation – using a combination of SARs and *in vitro* and short-term *in vivo* screening tests to determine if the substance has the potential to disrupt endocrine function in intact organisms. A weight of evidence approach is used with data from the three types of test, but short-term *in vivo* testing is considered essential.
 - ⇒ Confirmation – using a combination of subchronic and chronic *in vivo* tests to determine at what concentrations or doses endocrine modulating effects occur and, if possible, to elucidate more precisely the mechanisms of endocrine modulating effects (modes of action).
- Selection of *in vivo* tests at the screening and confirmatory levels should be exposure-driven, i.e. targeted at the environmental compartment or target organisms of concern.
- If a chemical is positively identified as an endocrine modulator, then a risk assessment should be performed following traditional approaches (e.g. comparison of predicted no effect concentration with predicted environmental concentration). However, EMs have potential for interactions as mixtures so great care should be used when considering substances individually.
- Triggers/criteria for moving through the strategy need to be developed.

The importance of international co-ordination of the efforts of competent authorities, industry, NGOs and academia in addressing issues relating to endocrine modulating effects in wildlife cannot be understated. This international co-ordination is being promoted by the Intergovernmental Forum on Chemical Safety and the Inter-organisation programme on the Sound Management of Chemicals.

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Table 1 Recommendations for *in vivo* testing at prioritisation/screening, subchronic and chronic levels

	Proposed Studies		
	Prioritisation/screening	Subchronic	Chronic
Mammals	<p>No existing tests are suitable, therefore recommend development of:</p> <ul style="list-style-type: none"> oestrogen/androgen screening system with an exposure period of 4-7 days. Very broad and can provide information on effects at level of gonad, hypophyseal and hypothalamic axis. additional integrative/short-term tests will also be needed to cover other modes of endocrine modulation. 	<p>Two suitable tests:</p> <ul style="list-style-type: none"> OECD 421: Reproduction/Developmental Toxicity Screening Test (using rat) OECD 422: Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test (using rat) <p>but with the following modifications:</p> <p>⇒ on adults: measurements of sperm production/motility, accessory sex organ weights, evaluation of oestrous cycle, epididymis, gonadal weight and histo-pathology, blood levels of sex hormones</p> <p>⇒ on offspring: developmental endpoints such as timing of vaginal opening and of preputial separation, anogenital distance, timing of testis descent, nipple development, gonadal weight, sperm production/sperm motility, accessory organ weights, evaluation of oestrous cycle.</p>	<p>Three suitable tests:</p> <ul style="list-style-type: none"> OECD 414: Teratogenicity OECD 415: One-Generation Reproduction Toxicity Study OECD 416: Two-Generation Reproduction Toxicity Study <p>but with the modifications also proposed for OECD 421 and 422.</p> <p>In addition, could take F1 generation in 415 up to time of vaginal opening.</p>
Birds	<p>No existing OECD Test Guidelines are suitable, therefore recommend two possible tests (using domestic fowl, mallard or Japanese quail):</p> <ul style="list-style-type: none"> gonadal development (gross effects, gonadal growth) and gonadal histopathology in subadults. Secondary sex changes (comb test and oviduct growth) could also be assessed. Start with exposure of 2-day old chicks, up to 14-20 days. A test is available and ready to implement, but would benefit from further development and appraisal. egg injection assays to measure effects on organisational and gonad development. Development of these tests needs further investigation. 		<ul style="list-style-type: none"> OECD 206: Avian Reproduction Test, but with modifications: <p>⇒ adults: preservation of accessory sex organs and gonads for future evaluation</p> <p>⇒ offspring (F1): accurate weight; gonadal weight/size, gross pathology, histo-pathology; sex ratio. Rear chicks to 8 rather than 14 days since endpoint of primary follicle formation in males is more prominent at this time.</p> <ul style="list-style-type: none"> recommend consideration of long-term multi-generation studies

Table 1 (continued)

	Proposed Studies		
	Prioritisation/screening	Subchronic	Chronic
Reptiles	None proposed	Hypothesis testing on a case-by-case basis	Hypothesis testing on a case-by-case basis
Amphibians	None proposed	Hypothesis testing on a case-by-case basis	Hypothesis testing on a case-by-case basis
Fish	No existing tests are suitable. Therefore recommend that a short-term test be developed with adult/subadult with an exposure period of 7-21 days, depending on the species. Possible species could include OECD test species (e.g. salmonids and cyprinids). Endpoints could include gonadosomatic index, plasma sex steroids, plasma vitellogenin (VTG).	No existing tests are suitable. Two possible methods were proposed, both need considerable development work: <ul style="list-style-type: none"> • adult fish exposed for 2-4 weeks (depending on species). Endpoints could include fecundity, steroid concentration, VTG production, sperm production. Possible species could include OECD test species (e.g. salmonids and cyprinids). • start exposure at most sensitive life stage (i.e. embryo or larvae) and continue up to and including gonadogenesis. Transfer fish to clean water to point of sexual maturity, then mate and measure: time to first batch of eggs, egg production, frequency of egg production, sex ratio, gonad histology. Possible species could include OECD test species (e.g. salmonids and cyprinids). 	Full life-cycle test (e.g. as in EPA guidelines). The high cost and technical difficulty of performing these studies is stressed.
Invertebrates	Test methods should be developed for this group. However, fundamental research on their endocrine systems needed first.		

Annex 1

Summary of relevant endpoints of existing OECD Test Guidelines (mammalian studies)

Endpoints identified (by life stage)	OECD Test Guideline								
	Acute			Subchronic				Neuro- tox	Geno- tox
Type of study	401	420	423	407	408	409	412	424	478
Preferred species	Rat	Rat	Rat	Rat	Rat	Dog	Rat	Rat	Rat/ mouse
Alternate species (if specified)	Other species (usually rodent)	Not specified	Other rodent	Other rodent	Other rodent	Primate or other (!)	Primarily rodent	Other rodent	-
Adult									†
General behaviour	X	X	X	X	X	X	X	X	X
Detailed behaviour/ function assessment					(X)	(X)		X	
Learning/memory assessment								O	
Gonadal weight/size	A	A	A	X-M A-F	X-M/ A-F(X)	X-M/ A-F(X)	X-M A-F		A
Gonadal gross pathological appearance	X	X	X	X	X	X	X	X	A
Gonadal histopathology	A	A	A	X	X	X	A		A
Accessory sex organ weight/size	A	A	A	X-M A-F	A(X)	A(X)	A		A
Accessory sex organ gross pathological appearance	X	X	X	X	X	X	X		A
Accessory sex organ histopathology	A	A	A	X	X-F A-M (X)	X-F A-M (X)	A		A

Table 2 (continued)

Summary of relevant endpoints of existing OECD Test Guidelines (mammalian studies)

Endpoints identified (by life stage)	OECD Test Guideline								
	401	420	423	407	408	409	412	424	478
Accessory sex organ secretory product production	A	A	A	A	A	A	A		A
Reproductive organ neoplasia									
Blood levels of sex hormones	A	A	A	≠	≠ (O)	≠ (O)	≠		A
Spermatogenesis (detailed histopathological assessment)	A	A	A	A	A	A	A		A
Sperm count/quality assessment	A	A	A	A	A	A	A		A
Oestrus cyclicity	A	A	A	A	A	A	A		
Time to mating									X
Mating/sexual behaviour									X
Gestation length									
<i>Corpra lutea</i> count									O
Litter pre-/post-implantation losses and abortion									X
Premature delivery									
Dystocia									
Fecundity (size/no. of litters)									X
Reproductive life span									
Maternal/lactational ability									
Offspring									
Sex ratio									
Sex differentiation									
Fetal weight									
Fetal skeletal abnormalities									
Fetal soft tissue abnormalities									
Sexual development and maturation (e.g. vaginal opening, preputial separation, etc.)									

Table 2 (continued)

Summary of relevant endpoints of existing OECD Test Guidelines (mammalian studies)

Endpoints identified (by life stage)	OECD Test Guideline								
	401	420	423	407	408	409	412	424	478
Growth rate/general development									
Learning and memory/behavioural development									
Gonadal weight/size									
Gonadal gross pathological appearance									
Gonadal histopathology									
Accessory sex organ weight/size									
Accessory sex organ gross pathological appearance									
Accessory sex organ histopathology									
Accessory sex organ secretory product production									
Blood levels of sex hormones									
Spermatogenesis (detailed histopathological assessment)									
Sperm count/quality assessment									
Oestrus cyclicity									
Time to mating									
Mating/sexual behaviour									

Table 2 (continued)

Summary of relevant endpoints of existing OECD Test Guidelines (mammalian studies)

Endpoints identified (by life stage)	OECD Test Guideline								
	401	420	423	407	408	409	412	424	478
Gestation length									
F ₂ litter pre-/post-implantation losses and abortion									
Premature delivery									
Dystocia									
F ₂ growth, development, etc.									

M Males F Females

≠ Option in Guidelines for hormonal assays, but does not specifically refer to sex hormones

X Guideline specifies parameter as endpoint that requires routine assessment

O Guideline indicates parameter as a possible (optional/or necessary) endpoint on a chemical/study-specific basis

A Endpoint which could be included in study design without significant disruption to methodology

* Duration of OECD 424 may be 28 or 90 days or greater than one year, depending on requirements

() Requirement different in revised draft version

! Use of primate not recommended in revised draft of OECD 409

† [ed?]

Table 2 (continued)

Summary of relevant endpoints of existing OECD Test Guidelines (mammalian studies)

Endpoints identified (by life stage)	OECD Test Guideline							
	Chronic/Carcinogenicity			Reproductive toxicity				
Type of study	451	452	453	414	415	416	421	422
Preferred species	Rat/mouse	Rat/dog/ primate	Rat	Rat/rabbit	Rat/mouse	Rat/mouse (rat)	Rat	Rat
Alternate species (if specified)	Hamster or other species	Not specified	Not specified	Mouse/ hamster (not specified)	Not specified	Not specified	Not specified	Not specified
Adult	□		□	Pregnant females				
General behaviour	X	X	X	X	X	X	X	X
Gonadal weight/size	A	X	X	A	A	A (X)	X-M A-F	X-M A-F
Gonadal gross pathological appearance	X	X	X	X	X	X	X	X
Gonadal histopathology	X	X	X	A	O	O (X)	X	X
Accessory sex organ weight/size	A	A	A	A (X)	A	A (X)	X-M A-F	X-M A-F
Accessory sex organ gross pathological appearance	X	X	X	X	X	X	X	X
Accessory sex organ histopathology	X	X	X	A	O	O (X)	X-M A-F	X

Table 2 (continued)

Summary of relevant endpoints of existing OECD Test Guidelines (mammalian studies)

Endpoints identified (by life stage)	OECD Test Guideline							
	451	452	453	414	415	416	421	422
Accessory sex organ secretory product production	A	A	A		A	A	A	A
Reproductive organ neoplasia	X	Possibly	X					
Blood levels of sex hormones	A	A	A		A	A	A	†
Spermatogenesis (detailed histopathological assessment)	A	A	A		O	O (X)	A	A
Sperm count/quality assessment	A	A	A		A	A	A	A
Homogenisation-resistant spermatids						(X)		
Epididymal sperm reserves						(X)		
Sperm motility/morphology						(X)		
Oestrus cyclicity	A	A	A		O	O (X)	A	A
Time to mating					X	X	X	X
Mating/sexual behaviour					X	X	X	X
Gestation length					X	X	X	X
<i>Corpra lutea</i> count				X				O
Litter pre-/post-implantation losses and abortion				X	X	X	X	X
Premature delivery				X	X	X	X	X
Dystocia					X	X	X	X
Fecundity (size/no. of litters)				X	X	X	X	X
Reproductive life span	A	A	A					
Maternal/lactational ability					X	X	X	X

Table 2 (continued)

Summary of relevant endpoints of existing OECD Test Guidelines (mammalian studies)

Endpoints identified (by life stage)	OECD Test Guideline							
	451	452	453	414	415	416	421	422
Offspring								
General behaviour						A (X)		
Sex ratio				X	X	X	X	X
Sex differentiation				X	X	X	X	X
Fetal weight				X				
Fetal skeletal abnormalities				X				
Fetal soft tissue abnormalities				X				
Sexual development and maturation (e.g. vaginal opening, preputial separation, etc.)						A (X)		
Growth rate/general development					X	X	X	X
Learning and memory/behavioural development					A	A (O)		
Gonadal weight/size					A	A (X)		
Gonadal gross pathological appearance					X	X	X	X
Gonadal histopathology					A	O (X)	A	A
Accessory sex organ weight/size					A	A (X)		
Accessory sex organ gross pathological appearance					X	X	X	X
Accessory sex organ histopathology					A	O (X)	A	A
Accessory sex organ secretory product production						A		

Table 2 (continued)

Summary of relevant endpoints of existing OECD Test Guidelines (mammalian studies)

Endpoints identified (by life stage)	OECD Test Guideline							
	451	452	453	414	415	416	421	422
Blood levels of sex hormones						A		
Spermatogenesis (detailed histopathological assessment)						A (X)		
Sperm count/quality assessment						A		
Homogenisation-resistant spermatids						(X)		
Epididymal sperm reserves						(X)		
Sperm motility/morphology						(X)		
Oestrus cyclicity						A (X)		
Time to mating						X		
Mating/sexual behaviour						X		
Gestation length						X		

Table 2 (continued)**Summary of relevant endpoints of existing OECD Test Guidelines (mammalian studies)**

Endpoints identified (by life stage)	OECD Test Guideline							
	451	452	453	414	415	416	421	422
F ₂ litter pre-/post-implantation losses and abortion						X		
Premature delivery						X		
Dystocia						X		
F ₂ growth, development, etc.						X		

- ‡ Option in Guidelines for hormonal assays, but does not specifically refer to sex hormones
- Treatment from pre-natal or natal stage sometimes appropriate
- ‡ Only male treated, mated with untreated virgin females
- X Guideline specifies parameter as endpoint that requires routine assessment
- O Guideline indicates parameter as a possible (optional) endpoint on a study-specific basis
- A Endpoint which could be included in study design without significant disruption to methodology
- () Requirement different in revised draft version

Table 3

Summary of relevant endpoints of existing OECD Test Guidelines (biotic systems)

Endpoints identified	OECD Test Guideline						
	202	203	204	205	206	207	210
Preferred species	<i>Daphnia magna</i>	See below	See below	See below	See below	<i>Eisenia foetida foetida</i>	See below
Alternate species (if specified)	Other daphnid	Other species	See below				
Free living/Fully adult form	Adult	Free	Free	Free	Adult	Adult	⊕
Survival	X	X	X	X	X	X	
Condition/size/abnormal behaviour	X	X	X	X	X	X	
Presence of eggs in brood pouch	X						
Egg production rate					X		
Number of cracked eggs					X		
Egg shell thickness					X		
Incidence of males	X						
Presence of winter eggs	X						
Gonadal weight/size					A		
Gonadal gross pathology					X		
Gonadal histopathology					A		
Accessory sex hormone weight/size					A		
Accessory sex organ gross pathology					X		
Accessory sex organ histopathology					A		
Blood sex hormone levels			A		A		

Table 3 (continued)

Summary of relevant endpoints of existing OECD Test Guidelines (biotic systems)

Endpoints identified	OECD Test Guideline						
	202	203	204	205	206	207	210
Offspring (F₁)							⊕
Embryonic stage at start							X
Hatching							X
Number of viable/non-viable eggs	X				X		X
Time to appearance of young/hatching	X						X
Time to end of hatching							X
Number of young	X				X		X
Survival of young					X		X
Condition/size/abnormal behaviour	X				X		X
Accurate weight							X
Accurate length							O
Gonadal weight/size					A		
Gonadal gross pathology					A		
Gonadal histopathology					A		
Accessory sex hormone weight/size					A		

Table 3 (continued)

Summary of relevant endpoints of existing OECD Test Guidelines (biotic systems)

Endpoints identified	OECD Test Guideline						
	202	203	204	205	206	207	210
Accessory sex organ gross pathology					A		
Accessory sex organ histopathology					A		
Blood sex hormone levels					A		
Detailed behaviour assessment					A		
Reproductive development and performance					A		
Subsequent Generations(s)							
As F ₁ generation	X						

- ⊕ OECD 210 performed on juvenile forms only (endpoints reported under offspring for convenience)
 X Guideline specifies parameter as endpoint that requires routine assessment
 O Guideline indicates parameter as a possible (optional) endpoint on a study-specific basis
 A Endpoint which could be included in study design without significant disruption to methodology

Table 3 (continued)

Summary of relevant endpoints of existing OECD Test Guidelines (biotic systems)

OECD 203/204: Recommended fish species - zebra fish (*Brachydanio rerio*), fathead minnow (*Pimephales promelas*), common carp (*Cyprinus carpio*), ricefish (*Oryzias latipes*), guppy (*Poecilia reticulata*), bluegill (*Lepomis macrochirus*) and rainbow trout (*Oncorhynchus mykiss*).

OECD 205: Recommended avian species - mallard duck (*Anas platyrhynchos*), bobwhite quail (*Colinus virginianus*), pigeon (*Columba livia*), Japanese quail (*Coturnix coturnix japonica*), ring-necked pheasant (*Phasianus colchicus*), red-legged partridge (*Alectoris rufa*).

OECD 206: Recommended avian species - mallard duck, bobwhite quail and Japanese quail.

OECD 210:

Recommended fish species:

- Freshwater: rainbow trout, fathead minnow, zebra fish and ricefish.
- Saltwater: sheepshead minnow (*Cyprinodon variegatus*).

Other suggested species:

- Freshwater: coho salmon (*Oncorhynchus kisutch*), chinook salmon (*Oncorhynchus tshawytscha*), brown trout (*Salmo trutta*), Atlantic salmon (*Salmo salar*), brook trout (*Salvelinus fontinalis*), lake trout (*Salvelinus namaycush*), northern pike (*Esox lucius*), white sucker (*Catostomus commersoni*), bluegill (*Lepomis macrochirus*), channel catfish (*Ictalurus punctatus*), flagfish (*Jordanella floridae*), three-spined stickleback (*Gasterosteus aculeatus*) and common carp.
- Saltwater: Atlantic silverside (*Menidia menidea*) and tidewater silverside (*Menidia peninsulae*).
-

Table 4

Summary of relevant endpoints proposed in draft OECD Test Guidelines (biotic systems)

Endpoints identified	OECD Test Guideline		
	211	212	Not assigned
Preferred species	<i>Daphnia magna</i>	Fish, see below	Fish, see below
Alternate species (if specified)	-	see below	see below
Free-living/Fully adult form		⊕	⊕
Survival	X		
Length (<i>i.e.</i> measure of growth)	O		
Time to first subsequent brood	O		
No./size of brood per animal	O		
No. aborted broods	O		
Presence of males	O		
Ephippia	O		
Condition/abnormal behaviour	A		

Table 4 (continued)

Summary of relevant endpoints proposed in draft OECD Test Guidelines (biotic systems)

Endpoints identified	OECD Test Guideline		
	211	212	Not assigned
Offspring (F₁)			
Stage of embryonic development		X	
Number of viable/non-viable young	X	X	X
Time to appearance of young/hatching	A	X	
Number of young	X	X	
Survival of young	X	X	X
Condition/size/abnormal appearance and behaviour	A	X	X
Accurate weight		X	X
Accurate length		X	A
Reproductive development and performance	A		
Subsequent generation(s)			
As F ₁ generation	A		

- ⊕ performed on embryos/juvenile forms only (endpoints reported under offspring for convenience)
X Guideline specifies parameter as endpoint that requires routine assessment
O Guideline indicates parameter as a possible (optional) endpoint on a study-specific basis
A Endpoint which could be included in study design without significant disruption to methodology

Table 4 (continued)**Summary of relevant endpoints proposed in draft OECD Test Guidelines (biotic systems)**

OECD 212: Recommended fish species:

- Freshwater: rainbow trout (*Oncorhynchus mykiss*); zebra fish (*Danio rerio*), previously described as *Brachydanio rerio* in OECD Documents); common carp (*Cyprinus carpio*); Japanese ricefish/Medaka (*Oryzias latipes*); fathead minnow (*Pimephales promelas*).

Other species used:

- Freshwater: goldfish (*Carassius auratus*); bluegill (*Lepomis macrochirus*).
- Saltwater: tidewater silverside (*Menidia peninsulae*); herring (*Clupea harengus*); cod (*Gadus morhua*); sheepshead minnow (*Cyprinodon variegatus*).

Unnumbered draft:

Recommended fish species:

- Freshwater: rainbow trout (*Oncorhynchus mykiss*).

Other species used:

- zebra fish (*Danio rerio*).

Summary of relevant endpoints proposed in draft OECD Test Guidelines (biotic systems)

Endpoints identified	OECD Test Guideline		
	211	212	Not assigned
Preferred species	<i>Daphnia magna</i>	Fish, see below	Fish, see below
Alternate species (if specified)	-	see below	see below
Free-living/Fully adult form		⊕	⊕
Survival	X		
Length (<i>i.e.</i> measure of growth)	O		
Time to first subsequent brood	O		
No./size of brood per animal	O		
No. aborted broods	O		
Presence of males	O		
Ehippia	O		
Condition/abnormal behaviour	A		

Table 4 (continued)

Summary of relevant endpoints proposed in draft OECD Test Guidelines (biotic systems)

Endpoints identified	OECD Test Guideline		
	211	212	Not assigned
Offspring (F₁)			
Stage of embryonic development		X	
Number of viable/non-viable young	X	X	X
Time to appearance of young/hatching	A	X	
Number of young	X	X	
Survival of young	X	X	X
Condition/size/abnormal appearance and behaviour	A	X	X
Accurate weight		X	X
Accurate length		X	A
Reproductive development and performance	A		
Subsequent generation(s)			
As F ₁ generation	A		

- ⊕ performed on embryos/juvenile forms only (endpoints reported under offspring for convenience)
 X Guideline specifies parameter as endpoint that requires routine assessment
 O Guideline indicates parameter as a possible (optional) endpoint on a study-specific basis
 A Endpoint which could be included in study design without significant disruption to methodology

Table 4 (continued)

Summary of relevant endpoints proposed in draft OECD Test Guidelines (biotic systems)

OECD 212: Recommended fish species:

- Freshwater: rainbow trout (*Oncorhynchus mykiss*); zebra fish (*Danio rerio*), previously described as *Brachydanio rerio* in OECD Documents); common carp (*Cyprinus carpio*); Japanese ricefish/Medaka (*Oryzias latipes*); fathead minnow (*Pimephales promelas*).

Other species used:

- Freshwater: goldfish (*Carassius auratus*); bluegill (*Lepomis macrochirus*).
- Saltwater: tidewater silverside (*Menidia peninsulae*); herring (*Clupea harengus*); cod (*Gadus morhua*); sheepshead minnow (*Cyprinodon variegatus*).

Unnumbered draft:

Recommended fish species:

- Freshwater: rainbow trout (*Oncorhynchus mykiss*).

Other species used:

- zebra fish (*Danio rerio*).

Annex**OVERVIEW OF NON REGULATORY TEST METHODS**

In order to assist in the comparison of the various non-regulatory models reviewed in this report, the assay principle, technical complexity, test durations and endpoints, together with assay sensitivity if provided, are summarised in Tables 6 and 7. Cell-free systems are not included in these tables, as their highly individualistic nature restricts the possibility of meaningful summarisation. Similarly, it was not possible to incorporate significant details for structure-activity relationship models. These are, however, discussed in detail in the Section Structure-Activity relationship modelling.

Table 6

Summary of duration and complexity of non-regulatory test methods

Test type	Model	Selectivity **	Animal	Approximate test duration			Specialised equipment or staff skills
				1 day	<1 week	>1 week	
Human-relevant - <i>in vivo</i>	Ovariectomised primate	O	Rhesus			Y	Surgery
	Uterotrophic assay	O	Rat, Mouse (possibly rabbit)	Y	Y	Y	Various procedures/endpoints; may involve surgery, complex biochemistry, image analysis, radioassay, etc; simplest is one-day study with uterine weight as endpoint
	Endometriosis	O	Rat & mouse			Y	Surgery
	Implantation assessment	G	Rat			Y	Very restricted endpoint
	Vagintrophic assay	G	Rat			Y	Chemical or surgical hypophysectomy; relatively complex dosing protocol
	Vagintrophic assay	O	Rat, mouse	Y	Y		Some models require ovariectomy; various available endpoints include: simple morphology/smears, weight, complex biochemistry
	Rodent mammary gland	O	Rat			Y	Immunocytochemistry/image analyser

Table 6 (continued)

Summary of duration and complexity of non-regulatory test methods

Test type	Model	Selectivity **	Animal	Approximate test duration			Specialised equipment or staff skills
				1 day	<1 week	>1 week	
Human-relevant - <i>in vivo</i> (continued)	“neoDES” mouse	O	Mouse			Y	Immunocytochemistry/ autoradiography/ gene expression
	Prenatal exposure/adult prostate model	O	Mouse			Y	Radioassay Surgery 3-D computer-assisted analysis
	Behavioural modification	G G O	Hamster Rat Mouse		Y Y	Y	Not yet fully developed as a test Not yet fully developed as a test None
	Cockscomb	A	Chicken		Y		
	Uropygial gland	A	Quail	Y		Y	Radioassay
	Submandibular gland	A	Rat			Y	Radioassay
	Prostate	A	Rat		Y		Radioassay/complex biochemistry Ultrasound scanner
		A	Dog			Y	
	Flank organ area	A	Hamster			Y	Surgery; possibly complex biochemistry
	Seminal vesicle weight	A	Mouse			Y	Surgery
	Sialic acid depletion	A	Dog			Y	Surgery
Chernoff & Kavlock assay	G	Mice			Y		

Table 6 (continued)

Summary of duration and complexity of non-regulatory test methods

Test type	Model	Selectivity **	Animal	Approximate test duration			Specialised equipment or staff skills
				1 day	<1 week	>1 week	
Human-relevant - <i>in vitro</i>	Mature/immature assay	A	Rat			Y	
	Primary endometrial cells	O	Human		Y		Human tissue handling; radioimmunoassay
	Primary ovarian epithelium	Not yet determined	Human				Not yet developed into a screening system
	T-74D cells	G	Human		Y		Varying complexity of equipment and complexity of calculations depending on endpoint
	ZR-75-1 cells	G	Human			Y	Interpretation of data obtained potentially complex
	MCF-7 cells	O	Human		Y		Simple cell counting in some assays; enzyme immunoassay kits for receptor levels in others; transfected cells require luminometer to measure activity of luciferase
	Ishikawa cells	O	Human		Y		Simple colour response in some models; others include autoradiography
	MDA-MB-231 CL10A cells	O	Human			Y	Transfection techniques

Table 6 (continued)

Summary of duration and complexity of non-regulatory test methods

Test type	Model	Selectivity **	Animal	Approximate test duration			Specialised equipment or staff skills
				1 day	<1 week	>1 week	
Human-relevant - <i>in vitro</i> (continued)	T-47D cells	O	Human			Y	Radioassay; immunoassay; complex biochemistry
	HeLa cells	O	Human		Y		Transfection techniques; radioassay; autoradiography; complex biochemistry
	Saos-2 cells	A	Human				Not yet developed into a screening system
	US-OS cells	A	Human				Not yet developed into a screening system
	Leydig cell culture	O	Mouse		Y	Y	Radioimmunochemistry; histochemistry
	Uterine cell culture	O	Rat		Y	Y	Radioassay; SDS-electrophoresis
	Pituitary cells	O	Rat			Y	Radioassay
	Hepa1c1c7 cells	G	Mouse		Y#		Transfection techniques
Embryonic fibroblast cells	O	Chicken		Y#		Transfection techniques	

Table 6 (continued)

Summary of duration and complexity of non-regulatory test methods

Test type	Model	Selectivity **	Animal	Approximate test duration			Specialised equipment or staff skills
				1 day	<1 week	>1 week	
Human-relevant - <i>in vitro</i> (continued)	L-cells	O	Mouse		Y#		Transfection techniques
	Uterus cells	G	Cow		Y		Radioassay; test underdeveloped
	Ovarian cells	G	Rat, mouse		Y	Y	Potentially demanding culture techniques; radioimmunoassay/ radioassay/electron microscopy test system (not yet fully developed)
	Embryonic hypothalamic cell	A	Mouse			Y	Radioassay
	PC 12 cells	A	Rat		Y#	Y	Transfection techniques; radioassay/specialised biochemistry/histopathological knowledge
	Insect cell line, Sf9	A	<i>Spodoptera</i>		Y#		Transfection techniques; complex biochemistry/autoradiography
	Yeast transfection models	A/O	<i>Saccharomy-ces cerevisiae</i>		Y#		Transfection techniques

Table 6 (continued)

Summary of duration and complexity of non-regulatory test methods

Test type	Model	Selectivity **	Animal	Approximate test duration			Specialised equipment or staff skills	
				1 day	<1 week	>1 week		
Structure-activity models	Mathematical modelling	A/O	-				Basic scientific knowledge not yet sufficiently developed for routine application	
Wildlife-relevant <i>- in vivo</i>	Fish toxicity test battery	G	Zebra fish		Y	Y		
	Long-term fish test	G	Medaka fish			Y	Extensive histopathology including electron microscopy and special staining of serial sections	
	Reproductive hormone	O	Goldfish		Y		Radioimmunoassay	
	Crustacean biochemistry	O	Daphnia		Y	Y	Radioassay	
	Sex reversal	G	A	Chicken			Y	Radioimmunoassay
				Alligator			Y	None
				Mosquito-fish			Y	None
	O		Tadpole			Y	Radioimmunoassay	
	O		Turtle			Y		
Vitellogenin induction	O		Various oviparous species		Y	Y	Principally radioimmunoassay or ELISA	
<i>Zona radiata</i> protein induction	O		Fish			Y	Western blot and ELISA assays	

Table 6 (continued)

Summary of duration and complexity of non-regulatory test methods

Test type	Model	Selectivity **	Animal	Approximate test duration			Specialised equipment or staff skills
				1 day	<1 week	>1 week	
Wildlife- relevant - <i>in vitro</i>	Vitellogenin induction	O	Trout		Y		Radioimmunoassay
	Oestrogen receptor binding	O	Sea trout		Y		Radioassay
			Turtle		Y		Radioassay
Androgen formation	A	Carp		Y		Radioimmunoassay	

** Test designed to detect:

A Androgens and/or anti-androgens

O Oestrogens and/or anti-oestrogens

G Other

Assumes transfected cell line already established and capable of storage until required

Y Yes

Table 7

Summary of endpoints and assay sensitivity of non-regulatory test methods

Test type	Model	Selectivity **	Animal	Endpoint (Sensitivity+)
Human-relevant - <i>in vivo</i>	Ovariectomised primate	O	Rhesus	Withdrawl bleeding Sexual skin colour
	Uterotrophic assay	O	Rat, mouse (possibly rabbit)	Uterine weight (2 µg/kg dose) Mitotic rate Peroxidase activity Ornithine decarboxylase Progesterone receptor Oestrogen receptor Fluid level Epithelial cell height
	Endometriosis	O	Rat, mouse	Multiple
	Implantation assessment	G	Rat	Implantation
	Vaginitrophic assay	G	Rat	Cornification
	Vaginitrophic assay	O	Rat, mouse	Vaginal opening Induction of oestrus
	Rodent mammary gland	O	Rat	Size Terminal ductal structure Proliferative index Labelling index

Table 7 (continued)

Summary of endpoints and assay sensitivity of non-regulatory test methods

Test type	Model	Selectivity **	Animal	Endpoint (Sensitivity+)
Human-relevant - <i>in vivo</i> (continued)	“neoDES” mouse	O	Mouse	Prostate weight Thimidine incorporation Receptor incidence Testicular and accessory sex organ weight Prostatic dysplasia c-myc-c-fos expression
	Prenatal exposure/ adult prostate	O	Mouse	Prostate weight 3-D computer assisted prostate reconstruction Hormone assay Receptor binding assay
	Behavioural modification	G G O	Hamster, rat, mouse	Urine marks (highly variable) Lordosis
	Cockscomb	A	Chicken	Comb ratio
	Uropygial gland	A	Quail	Receptor level C12-diol level
	Submandibular gland	A	Rat	Esteropeptidase activity 5 α -reductase & 5 α -dihydroxysteroid dehydrogenase activity 5 α -dihydrotestosterone nuclear binding

Table 7 (continued)

Summary of endpoints and assay sensitivity of non-regulatory test methods

Test type	Model	Selectivity **	Animal	Endpoint (Sensitivity+)
Human-relevant - <i>in vivo</i> (continued)	Prostate	A	Rat	Total binding sites Total weight DNA & RNA content
		A	Dog	Prostate cross-sectional area Testicular size Prostate histology
	Flank organ area	A	Hamster	Flank organ dimensions Sebaceous gland area Labelled glucose and lipid content of extract
	Seminal vesicle weight	A	Mouse	Weight wet /dry
	Sialic acid depletion	A	Dog	Sialic acid concentration
	Chernoff & Kavlock assay	G	Mice	Multiple teratogenic
Human relevant - <i>in vitro</i>	Mature/immature assay	A	Rat	Organ weights Serum hormone levels
	Pre-implantation embryo culture	O	Mouse	Embryo stickiness Appearance of zona pellucida
	Neural retinal cell	G	Chicken	Aggregation (highly variable) Growth (highly variable) Differentiation (highly variable)
	Primary endometrial cells	O	Human	Cell morphology Cell proliferation

Table 7 (continued)

Summary of endpoints and assay sensitivity of non-regulatory test methods

Test type	Model	Selectivity **	Animal	Endpoint (Sensitivity+)
Human-relevant - <i>in vitro</i> (continued)	Primary ovarian epithelium	Not yet determined	Human	Cell proliferation
	T-47D cells	G	Human	Alkaline phosphatase activity CAT reporter (0.1 nM progesterone)
	ZR-75-1 cells	G (multiple)	Human	Proliferation
	MCF-7 cells	O	Human	Cell proliferation (0.0001-1000% relative proliferative effect, based on E ₂) Thimidine incorporation (10 ⁻¹¹ M E ₂) Luciferase reporter (5 pg/mL E ₂) Exoprotein (0.0001-1000% effect relative to E ₂) Fluorescent assay of total DNA (0.1 nM E ₂)
	Ishikawa cells	O	Human	Alkaline phosphatase activity (10 ⁻¹² M E ₂) CAT/luciferase reporter
	MDA-MB-231 CL10A cells	O	Human	DNA measurement Fluorescence assay
	T-47D cells	O	Human	Multiple proliferative (EC ₅₀ 10 ⁻¹¹ M E ₂)
	HeLa cells	O	Human	Luciferase reporter CAT reporter

Table 7 (continued)

Summary of endpoints and assay sensitivity of non-regulatory test methods

Test type	Model	Selectivity **	Animal	Endpoint (Sensitivity+)
Human-relevant - <i>in vitro</i> (continued)	Saos-2 cells	A	Human	Receptor binding
	U2-OS cells	A	Human	Receptor concentration/receptor binding
	Leydig cell culture	O	Mouse	Androgen levels Steroid dehydrogenase levels
	Uterine cell culture	O	Rat	cGMP levels Receptor levels
	Pituitary cells	O	Rat	Electrophoretic profile (10^{-11} M E ₂) Prolactin levels
	Hepa1c1c7 cells	G	Mouse	Luciferase reporter (as equivalent to 80 pg TCDD per plate)
	Embryonic fibroblast cells	O	Chicken	CAT reporter (10^{-8} M E ₂)
	L-cells	O	Mouse	CAT reporter (10^{-10} M E ₂)
	Ovarian cells	G	Rat, mouse	Hormone levels; enzyme activities; hCG binding; histopathology
	Embryonic hypothalamic cell	A	Mouse	Aromatase activity
	Prostate androgen receptor	A	Rat	Relative binding affinity
	PC 12 cells	A	Rat	Multiple biochemical and visual
	Insect cell line, Sf9	A	<i>Spodoptera</i>	DNA binding of receptor
Yeast transfection models	A/O	<i>Saccharomyces cerevisiae</i>	β -galactosidase reporter (3 ng/L E ₂ ; EC ₅₀ 1 nM E ₂)	

Table 7 (continued)

Summary of endpoints and assay sensitivity of non-regulatory test methods

Test type	Model	Selectivity **	Animal	Endpoint (Sensitivity+)
Structure-activity models	Mathematical modelling	A/O	-	Functional similarity
Wildlife-relevant - <i>in vivo</i>	Fish toxicity test battery	G	Zebra fish	Spawning patterns (0.2-1 mg/L) Fertilisation Survival (0.04-1 mg/L)
	Long-term fish test	G	Medaka fish	Behaviour/gross anatomy (10mg/L β -hexachlorocyclohexane) Histopathology Fertilisation rates Spawning patterns
	Reproductive hormone	O	Goldfish	Plasma hormone concentration
	Crustacean biochemistry	O	Daphnia	Gross morphology and survival Steroid metabolism
	Sex reversal	G A O	Chicken Alligator Mosquito fish Tadpole Turtle	Phenotypic and biochemical markers of sex status Gonadal histopath Physical morphology Gross morphology Gonadosomatic index Phenotypic sex

Table 7 (continued)

Summary of endpoints and assay sensitivity of non-regulatory test methods

Test type	Model	Selectivity **	Animal	Endpoint (Sensitivity+)
Wildlife-relevant - <i>in vivo</i> (continued)	Vitellogenin induction	O	Various oviparous species	Plasma concentration (0.1 µg/mL) Plasma concentration of labelled vitellogenin (2 ng/mL)
	Zona radiata protein	O	Atlantic salmon	Plasma concentration
	Bursa fabricus development	G	Chicken	Weight
Wildlife-relevant - <i>in vitro</i>	Vitellogenin induction	O	Trout	Plasma concentration
	Oestrogen receptor binding	O	Sea-trout Turtle	Receptor concentration (10 ⁻¹¹ -10 ⁻³ M) Specific binding
	Androgen formation	A	Carp	Androgen concentration (0.25 pg/mL)

** Test designed to detect:

A Androgens and/or anti-androgens

O Oestrogens and/or anti-oestrogens

G Other

+ Assay sensitivity data only presented where specifically defined by author

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