

***THE USE OF METABOLISING SYSTEMS FOR IN
VITRO TESTING OF ENDOCRINE DISRUPTORS***

A DETAILED REVIEW PAPER

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TABLE OF CONTENTS

	Executive Summary	5
1	Endocrine Disruption	10
1.1	Introduction	10
1.2	Mode of action of ED's	14
1.3	The need for <i>in vitro</i> test for endocrine disruption	18
1.4	Test for endocrine disruptors <i>in vitro</i>	18
1.4.1	<i>In vitro</i> receptor binding assays	20
1.4.2	Induction of hormone responsive mammalian cell proliferation	21
1.4.3	Transactivation assays (hormone-sensitive transcription of reporter genes)	22
2	Metabolism	23
2.1	The role of metabolism in activation and detoxification of xenobiotics: Phase 1 and Phase 2 metabolism	23
2.2	<i>In vitro</i> methods for the measurement of metabolism	26
2.2.1	Current status of computer-based approaches for assessing metabolism	26
2.2.2	Types of metabolising systems applied to <i>in vitro</i> test systems	28
2.3	The relevance of metabolism to the testing of endocrine disruptors	33
2.4	Potential problems with using exogenous metabolism to studies of endocrine disruptors	42
2.5	Fetal metabolism	44
2.6	Metabolism during digestion	45
3	The metabolism of endogenous steroids	46
3.1	Introduction	46
3.2	Metabolic activation of endogenous steroids	49
3.3	Metabolic inactivation of endogenous steroids	50
3.4	The consequences of metabolism for hormonal activity	52
4	The testing of endocrine disruptors and metabolism	52
4.1	Introduction	52
4.2	Possible ways to overcome problems of using exogenous metabolising systems	54
4.2.1	The intrinsic metabolising capacity of cell lines used for testing endocrine disruptors	55
4.2.2	The potential to use genetically-engineered cell lines	56
5	Proposals to develop a framework for the simultaneous testing of endocrine disruption and metabolism	58
5.1	Tiered approach including determination of the likelihood of systemic exposure	58
5.2	<i>In vitro</i> methods to study metabolism	58
5.2.1	Screening tests for metabolism	59
5.2.2	Induction of biotransformation enzymes	61

5.2.3	Models for evaluating polymorphic effects on metabolism	62
5.3	Approach to combine the <i>in vitro</i> study of endocrine disruption and metabolism	63
6	Conclusions and recommendations	68
7	References	71
8	Tables	114
9	Figures	123

Abbreviations used:

AhR: Aryl hydrocarbon receptor
AR: Androgen receptor
ARNT: AhR Nuclear Translocator
CAR: Constitutive Androstane Receptor
CAT: Chloramfenicol transferase
CYP: cytochrome P450
DNA: Deoxyribonucleic acid
ED: Endocrine disruptor
ER: Estrogen receptor
ECVAM: European Centre for the Validation of Alternative Methods
EDSTAC: Endocrine Disruptor Screening and Testing Advisory Committee
EPA: Environmental Protection Agency (U.S.)
FSH: Follicle-stimulating hormone
G: General *in vivo* tests with potential endocrine endpoints (OECD 407 (enhanced), 415, 416)
GFP: Green fluorescent protein
hEST: human estrogen sulphotransferase
HRE: Hormone response element
HPLC: High performance or high pressure liquid chromatography
ICCVAM: Interagency Coordinating Committee on the Validation of Alternative Methods
LH: Luteinizing hormone
M1: 2-[[3,5-dichlorophenyl-carbamoyl]oxy]-2-methyl-3butenoic acid
M2: 3',5'-dichloro-2-hydroxy-2-methylbut-3-enamide
MC: Assessment of metabolism of the chemical under study *in vitro*
MH: Assessment of metabolism of hormones *in vitro*
NADPH: Nicotinamide adenine dinucleotide phosphate (reduced form).
NICEATM: National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods
NRs: Nuclear Receptors
OECD: Organisation for Economic Co-operation and Development
P: Primary test for endocrine disruption *in vitro*
P450: cytochrome P450
PCB: Polychlorinated Biphenyl
PBDE: Polybrominated Diphenyl Ether
PMC: Test combining a primary test for endocrine disruption *in vitro* and for metabolism of the chemical under study
PMH: Test combining a primary test for endocrine disruption *in vitro* and for metabolism of the chemical under study
PHS: prostaglandin H synthase system
PXR: Pregnane X Receptor (also termed SXR: Steroid and Xenobiotic Receptor)
(Q)SAR: (Quantitative) Structure Activity Relationship
(Q)SPR: (Quantitative) Structure Property Relationship
SHR: Steroid Hormone Receptor
SULT: sulphotransferase

EXECUTIVE SUMMARY

Legislation or draft proposals in for instance Europe, Japan and the USA require that chemicals are tested for their ability to disrupt the hormonal systems of mammals. Such chemicals are hormonally-active and are known as endocrine disruptors (EDs). A widely accepted definition of an ED was proposed in 1996 in Weybridge: “An endocrine disruptor is an exogenous substance that causes adverse health effects in an intact organism, or its progeny, secondary to changes in endocrine function. The word “secondary” has sometimes been replaced by “consequent”. The existing evidence about endocrine disrupting activity is mostly related to chemicals that interfere with estrogenic or androgenic mechanisms, or that interfere with thyroid hormone function. Therefore the focus of this paper will be on these particular hormonal systems, but other hormones may turn out to be important in the future. Both *in vivo* (whole animal-based) and *in vitro* (non-whole animal based) tests are currently being proposed for the testing of endocrine disrupting activity. Animal tests are justified on the basis that it is impossible to model the complex responses of the whole body to EDs, including biotransformation. To date, there is still little or no experience with incorporating metabolic and toxicokinetic aspects into *in vitro* tests for EDs. This is a situation in sharp contrast to other areas of toxicity testing such as the genotoxicity area where *in vitro* tests are routinely conducted with and without metabolic capacity. This Detailed Review Paper aims to justify why *in vitro* assays for EDs should incorporate mammalian metabolising systems and to indicate how this could be done. The proper tests to detect EDs are still being developed or being validated, and tests that can predict the influence of chemicals on metabolism of endogenous or exogenous substances, or the influence of metabolism of a chemical on its ultimate effect, are not yet accepted for general use either. Therefore it will be difficult at present to incorporate measurements of metabolism into *in vitro* testing for endocrine disruption within the context of validated tests. However, some existing tests that take metabolism into account could perhaps already be used to prioritize substances for further testing.

The background to ED testing, the available test methods, and the role of mammalian metabolism in the activation and the inactivation of both endogenous and exogenous steroids

is described. The available types of metabolising systems are compared, and the potential problems in incorporating metabolising systems in *in vitro* tests for EDs, and how these might be overcome, are discussed. Lastly, some recommendations for future activities are made.

EDs act by a diversity of mechanisms including receptor binding, altered post-receptor activation, altered hormone synthesis, hormone storage, and release, clearance and perturbation of homeostasis. The main *in vitro* tests for EDs comprise subcellular hormone receptor ligand binding, the induction of proliferation (mitogenesis) in hormone-responsive mammalian cell lines, and transactivation systems in yeast and mammalian cell lines. The latter systems involve genetically-engineered cells which carry DNA sequences for mammalian hormone receptors, in conjunction with the respective response elements linked to promoter regions, together with the reporter gene itself. For the time being, ICCVAM has recommended not using yeast based assays or proliferation assays for ED testing because of potential problems with the penetration of substances through the cell wall and because it is not possible to observe the differences between agonists and antagonists. However, there is also a wealth of ED data generated by these systems in the public domain that, with normalization, might be usefully incorporated into multivariate and (Quantitative) Structure Activity Relationships ((Q)SAR) ED analyses. Furthermore, it might be worthwhile to investigate the possibilities to incorporate metabolic capacity in this type of cells by genetic engineering techniques and to determine the characterisation and intrinsic metabolic capacity of the cells used in proliferation and transfection assays.

It is well-established that information on the metabolism of a substance is important in the evaluation of its toxic potential. Such metabolism occurs especially in the liver, but also in extrahepatic tissues, and is divided into phases I and II. The former involves the mixed function oxidase activities of the many isoforms of the cytochrome P450 (CYP) family of enzymes. Phase II metabolism involves the conjugation of metabolites generated by oxidation reactions in Phase I to water soluble entities. In extra-hepatic tissues, the prostaglandin H synthase system is particularly active and causes the oxidation of a wide

range of xenobiotics. In addition, metabolism by certain mixed function oxidases must also be considered in extra-hepatic tissue (Bernauer et al., 1999; 2000; 2002; 2003a) and it has to be taken into consideration, that extrahepatic metabolism may differ from hepatic metabolism. Metabolising systems for use *in vitro* can be either subcellular (enzyme homogenates, cytosol, microsomes) or cellular (the residual metabolism of the indicator cells themselves, metabolically competent primary cells or genetically-engineered cell lines). Despite the fact that there are several technical problems with using sub-cellular metabolising systems in assays for testing EDs (such as non-specific binding to protein, potential toxicity to target cells), there is sufficient evidence in the literature to show that these limitations can be overcome, and that the presence of such enzymes can modulate the chemical toxicity of several types of EDs.

An initial recommendation is that future testing should first aim at determining whether a substance is likely to be systemically available, with special attention to the oral absorption in neonates. The use of (Q)SAR and (Quantitative) Structural Property Relationships ((Q)SPR) to select chemicals that may have ED effects and/or that could be metabolised or interfere with metabolism of endogenous and exogenous substances should be explored.

There is a good deal of information showing that endogenous steroids are extensively metabolised by phase I and II enzymes in both the liver and their target tissues, and that such metabolism can lead to the inactivation of steroids. In the case of endogenous steroids such reactions will affect their abilities to perform their normal functions. Obviously exogenous compounds that interfere with the inactivation of natural hormones can thereby cause endocrine disrupting effects.

Inadequate incorporation of metabolism into *in vitro* tests for endocrine disruption could give rise to false positive data (due to lack of detoxification) or false negative data (due to lack of bioactivation) arising from the use of the tests. As yet, there is little information on the intrinsic metabolising potential of cell systems traditionally used to detect EDs. It is recommended to obtain this information as quickly as possible. No cell line is available at

present that is genetically-engineered to express phase I, II or I and II enzymes simultaneously with molecular targets for detection of ED activity. While examples of such cell constructs are known to be currently under development by at least two different research groups (Elcombe C., and Lichtenberg-Fraté H., personal communications), it is suggested to start a feasibility study into the generation of genetically-engineered mammalian and yeast cell lines containing steroid hormone nuclear receptors, their response elements and reporter genes, together with genes expressing both phase I and II metabolising enzymes now. Such cell lines expressing multiple molecular entities may have the advantage that there will be less binding to exogenous protein than might be the case when utilizing enzyme fractions, and furthermore, metabolites formed may not need to be transported from their sites of formation to intracellular targets. Recent developments with cytochrome P450 and receptor insertion into genetically modified yeast cell lines also show promise (Lichtenberg-Fraté H., personal communication, and Siervernich et al 2004). If these developments are confirmed, they should be developed into tests that can be used for regulatory purposes.

It is further recommended that there should be an assessment of the intrinsic metabolising capacity of cell systems used in tests for EDs and an investigation into the relevance of using the prostaglandin H synthase (PHS) system and other, non-cytochrome P450 dependent enzymes, for metabolism of EDs.

Much could be learned from the areas where metabolism is already incorporated like genotoxicity testing and the testing of cosmetics to design tests that could combine the assessment of endocrine effects and metabolism effects. Possibilities to make quick progress are to incorporate an S9 fraction into the *in vitro* tests for endocrine disruption, as is the case for genotoxicity and mutagenicity testing and to explore the possibilities to make use of the tiered approach that was proposed for the testing of cosmetics. The use of these methods in ED screening assays would imply that the potential toxicity that S9 could cause to target cells and the problem of potential protein binding need to be carefully addressed and well controlled.

It is concluded that there is no justification for metabolism to be excluded from *in vitro* tests for EDs, and the perceived difficulties of incorporating metabolism into *in vitro* tests for EDs should not deter more research into how this can be achieved. The development and validation of these *in vitro* assays in conjunction with appropriate metabolising systems should be a matter of urgency. For the time being, a framework is proposed in order to incorporate already as much as possible the testing of endocrine disrupting activity coupled to assessment of the role played by metabolism *in vitro*, while tests are still being standardised and validated. The tests for ED targets and for metabolism can be developed and validated separately, but it is advisable that from the early phases on, attention is given to the fact that they ultimately may need to be combined.

1. Endocrine disruption

1.1 Introduction

It is widely believed that exposure to natural and synthetic chemicals in the environment, with potential to interfere with the endocrine system, may elicit a wide range of toxic effects in wild-life and in humans, in particular on the reproductive system and with respect to the induction of cancer (Atterwill & Flack, 1992, Neubert, 1997; McLachlan 2001; Baker, 2002; Eertmans *et al.*, 2003). Such chemicals are termed endocrine disruptors (EDs). Different definitions for EDs have been proposed but one that is widely accepted was given at a conference in Weybridge (1996): “An endocrine disruptor is an exogenous substance that causes adverse health effects in an intact organism, or its progeny, secondary to changes in endocrine function. The word “secondary” has sometimes been replaced by “consequent”. Recently, interest has focused on exogenous substances, which have come to be called endocrine disruptors (EDs), which are thought to induce sex steroid-like effects (Harvey *et al.*, 1999). Some of these chemicals are structurally-related to endogenous steroids while others are not (McLachlan. 2001). Endogenous sex steroid hormones, such as estrogen, testosterone and progesterone are a class of chemicals characterised by the presence of a 4-ringed nucleus possessing high lipid solubility. Other related substances include the corticosteroids, mineralocorticoids, vitamin D and retinoic acid. Although the D vitamins are not steroids, all these substances are known to act on nuclear receptors of the same superfamily. Also thyroid hormone is known to act on nuclear receptors that belong to the same superfamily although they are structurally different from the steroid hormones (Orti 1992; Graham and Clarke 1997, McLachlan 2001; Nilsson *et al.* 2001).

Up to now most attention with regard to endocrine disruption has until now been focused upon sex steroid hormones and thyroid hormone because most evidence for such activity has been produced in these domains and as a consequence also tests have been made available that focus on these targets (EDSTAC 1998). There is no reason why other hormones like other steroid hormones, Follicle Stimulating Hormone (FSH), Luteinising Hormone (LH), insulin, insulin like growth factors, growth hormone etc., could not be affected by exogenous

chemicals. Although it may be advisable to explore these possibilities, at present it seems to be premature to include them in a screening strategy with regulatory implications. This Detailed Review Paper will therefore focus attention to the hormones for which there is currently evidence that they are affected by xenobiotics. The mechanisms that may be involved include:

- specific binding to one or more members of an extensive family of hormone nuclear receptors (NRs)
- interaction with hormone receptors or hormone mediated transcription
- inhibition of steroidogenic enzymes
- chemically induced inhibition or induction of hormone metabolism
- other mechanisms.

Legislation in the USA requires the testing of chemicals for endocrine activity (the *Food Quality Protection Act* of 1996, Public Law 104-170, and *Safe Drinking Water Act Amendments* of 1996, Public Law 104-182; Anon, 2001a). Other regions of the world, especially Japan and Europe, are also introducing or discussing legislation for ED testing (see e.g. Anon, 2001b). The specific testing of chemicals for ED activity may be incorporated into the new REACH legislation being developed by the EC (Combes *et al.*, 2003), although at present endocrine disruption is considered by the European Union as a mechanism that could have an impact on endpoints in general toxicology testing and not as an endpoint in its own right.

In the USA, the EPA was charged with developing and validating a testing strategy for EDs. For this purpose, the EPA established an Endocrine Disruptors Screening and Testing Advisory Committee (EDSTAC) to consider possible testing strategies for EDs. EDSTAC concluded that there is a need to include both *in vitro* and *in vivo* assays in a tiered approach. The proposed Tier 1 screening phase comprises both *in vitro* and *in vivo* assays, aimed primarily at detecting chemicals with endocrine activity and thus potential human health effects. Among the recommended *in vitro* assays included in the battery are cell-free receptor binding, transcriptional activation, steroidogenesis in testis or Leydig cell cultures

(despite reservations expressed about this particular assay), and frog metamorphosis. The Tier 2 test battery solely consists of *in vivo* assays, and includes two generation reproductive mammalian tests, as well as tests for environmental effects on target wild-life species. It is intended that the Tier 2 battery will provide the definitive data on EDs suitable for risk assessment purposes.

An OECD Working Group on Endocrine Disruptor Testing and Assessment (EDTA) has been established to define tests for validation. As part of this activity, the OECD, in conjunction with the US Environmental Protection Agency (EPA), has become directly involved in validation programs for new animal tests (Combes and Balls, 2003). For this purpose, the OECD has established a group to manage the validation of three new animal methods for the detection of EDs, namely, a uterotrophic assay for estrogens and anti-estrogens, the Hershberger test for androgens and anti-androgens, and an enhanced 407 protocol for repeat dose toxicity to detect agents acting via these and other mechanisms, such as steroidogenesis. While the development, standardization and validation of *in vitro* tests to detect endocrine disrupting activity is in progress and Detailed Review Papers on steroidogenesis and aromatase activity are being drafted, the Validation Management Group for non-animal tests within the EDTA has expressed concern that metabolism of xenobiotics is not yet considered for incorporation into the *in vitro* tests that will be used in the future and in particular with regard to endocrine disruption. In preparing an effort to combine metabolism and ED screening, the aim of the present Detailed Review Paper is to review the available evidence and to suggest possibilities to reach this goal. The methods for *in vitro* measurement of metabolism still need a lot of development, standardization and validation. ECVAM has addressed the issue with regard to cosmetics and has also held a workshop to review the possibilities for *in vitro* testing of metabolism in a more general context. These efforts generated much information that is also relevant to endocrine disruption testing *in vitro* and hopefully the research resulting from these workshops will solve a lot of the existing shortcomings with regard to endocrine disruptor testing.

This DRP paper will especially focus on introducing metabolic competence into the *in vitro* test design. But testing for endocrine disruption has to be considered, as with all potency

tests which will be used in hazard and risk assessment, to be a building block in an overall biokinetic evaluation of the endocrine disrupting potency.

Biokinetic processes, like absorption, distribution, biotransformation and excretion, determine the relation of the administered and the internal dose. For *in vivo* animal studies, regulatory toxicokinetic studies determine the relation between the administered dose via the oral, dermal or inhalation route and the internal dose. When carrying out *in vitro* studies the same biokinetic information will be crucial to relate the administered *in vitro* dose to the internal dose (i.e. free concentration) reaching the cellular targets. Furthermore, it will be essential to relate the *in vitro* dose-regime and the bioavailability of the parent compound and its possible metabolites to a relevant dose *in vivo*. Therefore, the existing *in vivo* studies on ED compounds will be important to obtain realistic *in vitro* experimental designs for determining ED potency.

A tiered approach is recommended to be followed in order to obtain all the necessary information to conduct a hazard and risk assessment, based on *in silico* and *in vitro* data sets. For any compound, information is needed on its likelihood to be systemically available, depending on the route of exposure. Furthermore, once entered into the system, the compounds' distribution and also their biotransformations have to be evaluated. Once physiologically-based biokinetic models have been developed for the specific purpose to be used in a regulatory context, predictions will be possible concerning target system and target organ distribution of compounds and the importance of biotransformation aspects.

The intention of this DRP is not to duplicate the work that is already being done in the development and validation of tests for endocrine disruption (ICCVAM 2003), and also being conducted by ECVAM, or for kinetics and metabolism of chemicals (ECVAM 2003, 2004) separately, but rather to offer a framework to bring the two approaches together. Although interference of chemicals with steroidogenic enzymes and aromatase may be a major pathway towards ED activity, this will not be dealt with in detail because they are already the subject of a separate DRP at the OECD.

A frequently mentioned hypothesis with regard to environmental contaminants and in particular EDs, is the possibility that low concentrations of different chemicals (which may not be effective alone) may interact, resulting in additive (Silva et al. 2002) or even synergistic effects. However, although it is realistic to assume that exposure to mixtures is what happens in everyday life, it would be out of the scope of this DRP to address the question how metabolism could be incorporated in *in vitro* models to study the action of mixtures. Indeed the incorporation of metabolic capacity into tests to evaluate single chemicals is complex in itself and adding a further degree of complexity at this stage does not seem to increase the chances of developing and validating new tests quickly. It would also not be obvious to decide which mixture of chemicals is actually going to mimic real exposures best, because infinite variations are possible. Furthermore, the tests that need to be developed will be used to investigate single compounds for regulatory purposes. It is therefore considered as a first priority to develop methods that would allow the study of single chemicals.

1.2 Mode of action of EDs

EDs act by a diversity of mechanisms including receptor binding, altered post-receptor activation, altered steroidogenesis (modulation of hormone synthesis), perturbation of hormone storage, release, clearance and homeostasis. The immediate effects of these activities, depending on the nature of the ED can be for instance: estrogen agonism or antagonism, androgen agonism or antagonism, suppression or induction of levels of other endogenous hormones, such as the release of FSH, LH or prolactin, or other effects that are the consequence of these changes.

Since many mechanisms exist through which EDs can exert an estrogenic or androgenic agonist effect, the terms estrogenic/anti-estrogenic or androgenic/anti-androgenic do not always have the same meaning when used in the literature. Usually estrogenic or androgenic means that a chemical has the same effect as 17 β -estradiol or testosterone or another recognised natural or synthetic agonist acting at the ER or AR in a particular system. Anti-estrogenic or anti-androgenic means that a substance prevents 17 β -estradiol or testosterone

or another recognised natural or synthetic agonist acting at the ER or AR from exerting its effect in a particular model.

The effects mentioned above are mediated in part by binding specifically to an extensive family of nuclear receptors (steroid hormone receptors or SHRs; Warner *et al.*, 1999). SHRs have structural features in common which include a central highly-conserved DNA binding domain that targets the receptor to specific DNA sequences (hormone response elements, HREs). The terminal portion of the receptor includes a ligand binding domain that interacts directly with the hormone. This domain contains a hormone-dependent transcriptional activation region. Binding of the steroid hormone to the receptor protein causes it to undergo a conformational change resulting in binding of the DNA to the HRE to form a complex that triggers or suppresses the transcription of a specific set of genes (Hornhardt and Wiebel 1996; Wiegel, 1996; Jacobs and Lewis, 2002; Nilsson, 2001 and Combes, 2000a).

The estrogen receptor (ER) exists in at least 2 subtypes, termed α (the first discovered) and β , and each of these is encoded by a separate gene (Orti *et al.* 1992; Graham and Clarke 1997, McLachlan 2001; Nilsson *et al.* 2001). These ER subtypes differ in their tissue distribution and relative ligand binding affinities for the same hormones (a fact that could account for differential hormonal effects depending on the tissue) and also appear to have complementary but different functions (reviewed in Jacobs 2005). ER β , has been shown to be antiproliferative when present along with ER α in breast cancer cells (Paruthiyil *et al.* 2004), although the role of ER β in breast cancer is unclear. It should be noted that apart from these two nuclear receptors there has been identification of a γ subtype in teleosts that appears to have evolved from ER β gene duplication and may mediate some effects of EDs in these fish (Hawkins *et al.* 2000; Hawkins and Thomas 2004). Furthermore, there is an estrogen receptor that is located in the cell membrane which is responsible for the fast non-genomic responses to the hormone (McEwen and Alves 1999; Belcher and Zsarnovsky 2001; Matthews and Gustafsson 2003; Hasbi *et al.* 2005). While the ER located in the cell membrane seems to be structurally related to the nuclear ERs, they can

also be activated by larger molecules that are unable to enter the nucleus and bind with the nuclear ERs (Harrington et al 2005). However, the possibility that cofactors or the immediate surroundings of the receptor may influence binding affinity differently, that signal transduction mechanisms coupled to membrane receptors can be affected by chemicals in a different way from the NRs, and the fact that chemicals that do not easily penetrate into cells can reach the membrane receptors, may need to be taken into account. Most studies have been directed to the α sub-form of the ER, and therefore combined assessments of metabolism and interference with estrogenic mechanisms should include this subtype. ER α stimulates a classic estrogenic response; ER β modifies this by altering the recruitment of c-Fos and c-Jun to estrogen responsive promoters (Matthews et al. 2006). Currently ER α is the ER being (pre) validated for ER binding and activation hazard assessment screening procedures, but whether addressing only ER α binding is sufficient for regulatory screening purposes requires further investigation.

A similar binding affinity was for instance suggested by one study with a selection of industrial chemicals but this was not confirmed in the same study with phytoestrogens (Kuiper et al. 1998) and several later studies have confirmed the selectivity of phytochemicals and their metabolites for ER β (e.g. De Angelis et al 2005) . In particular with regard to the efficacy to induce effects, but also perhaps with regard to ligand binding affinity, substances can behave differently on both subtypes (Barkhem et al. 1998). Metabolic competence may need to be included in all ER-related *in vitro* tests that will ultimately be deemed necessary for ED testing.

The interest in thyroid hormone has arisen because it plays a role in the control of development (e.g. metamorphosis in amphibians and brain development in mammals), and chemicals may alter its synthesis, transport and catabolism (Capen 1997; Cheek et al. 1999; Craft et al. 2002; DeVito et al. 1999; Fowles et al. 1994; Howdeshell 2002; Kato et al. 2000; Zhou et al. 2001). Interference by xenobiotics with the turnover over the thyroid hormone may at present seem to be a more important mechanism for causing endocrine disruption than interactions at the thyroid receptor level. However, PCB's may interfere with the thyroid receptor function and some of these chemicals seem to suppress transcription that is

linked to thyroid receptor activation (Miyazaki et al. 2004; Fritsche et al. 2005). Thyroid receptor binding assays or transactivation assays incorporating metabolism may therefore be needed. Different thyroid hormone receptors were described (Lazar 1993; Yen 2001) and the question which of them needs to be used in binding or transactivation assays may need to be addressed sooner or later. With regard to the androgen receptor, the issue of different subtypes to be tested does not exist at present.

The Aryl hydrocarbon receptor (AhR) is also involved in modulating the effects of retinoids (Soprano and Soprano 2003), estradiol and dexamethasone (Lai et al. 2003) or progesterone (Kuil et al. 1998). Thus, a mouse hepatoma cell line (Hepa1c1c7) possessing this receptor, in conjunction with a dioxin response element and a luciferase reporter gene complex, was shown to respond to chemicals with dioxin-like activity in pulp-mill effluents (Zacharewski *et al.*, 1995). The AhR, a member of the PAS (Per-Arnt-Sim) family of nuclear regulatory basic helix-loop-helix proteins, has been detected in nearly all vertebrate groups examined (Hahn 1998; Hahn 2002; Kato et al. 2002) and appears to have a fundamental role in cellular physiology, neurodevelopment and circadian rhythmicity (Poellinger 2000). Upon ligand binding the receptor dissociates from heat shock protein with which it forms a complex in the cytosol, it enters the nucleus and joins with the heterodimeric partner AhR Nuclear Translocator ARNT (Kato et al. 2002). Predominantly found in hepatocytes, but also in breast cancer cells (Nguyen et al. 1999), and the lung (Song et al. 2002), this AhR/ARNT dimer interacts with the Dioxin Response Elements (DRE) on the DNA, resulting in ligand dependent transcription of CYP1A1, CYP1A2, CYP1B1, the glutathione S-transferase subunit Ya, quinone oxidoreductase, UDP-glucuronosyltransferase and aldehyde-3-dehydrogenase (Kato et al. 2002). If chemicals interfere with the activity of the AhR, they may thus have effects on metabolism of xenobiotics, including these chemicals themselves, and on the concentration of steroid hormones, which would lead to endocrine effects. For instance the *in vitro* antiestrogenic activity in MCF-7 breast tumor cells caused by 2,3,7,8-tetrachlorodibenzo-p-dioxin is explained by enhanced metabolism of estradiol through this pathway (Spink et al. 1990). ER-mediated estrogen signalling may also be modulated by a co-regulatory-like function of activated AhR/ARNT, giving rise to adverse estrogen-related

actions of dioxin-type contaminants (Ohtake et al. 2003). Such a complex mechanism may be extremely difficult to investigate *in vitro*, but a simple Ah receptor assay may be able to pinpoint a number of chemicals that need further investigation with respect to interference with metabolism.

1.3 The need for *in vitro* tests for EDs

Animal tests for detecting endocrine disruptors are necessarily detailed, since many mechanisms can be involved in the perturbation of the complex hormonal systems of mammals, and there are subtle inter-species differences in the ways these work and the consequences in terms of physiology, reproduction and development (Zacharewski, 1998). This means that the validation of these assays needs very careful consideration (Ashby, 2000).

The EDSTAC Tier I testing scheme requires that both *in vitro* and *in vivo* screens should be developed, and it is essential that the former test methods are developed and validated so that they can be used first in the test battery for prioritizing chemicals for further testing and also to facilitate the interpretation of data from animal tests.

From an animal welfare point of view, potentially very large numbers of animals will be required for endocrine disruptor testing, should the current EPA proposals go ahead. The actual numbers will depend on the sensitivity and specificity of the testing methods that will be used (Purchase, 1999). The use of animals is morally controversial, and often more time-consuming and costly than using non-animal approaches. The reduction of animal testing is an important reason to develop and validate *in vitro* models for testing EDs that include metabolism of the chemicals to be tested.

1.4 *In vitro* tests for endocrine disruptors

There have been several studies on the structure-activity of EDs (e.g. reviewed in Combes, 2000; Benfenati E et al. unpublished review report for the European Chemicals Bureau,

European Commission, December 2005) most of which have been conducted for the ER α . In summary it appears that many natural and synthetic estrogens, generally have in common the phenolic OH group on a small lipophilic molecule of about 200 - 300 Daltons. However, the OH substituent is not essential for ER mediated activity. There is a large diversity of potential EDs and many possible molecular targets through which they might act. The possibility that (Q)SARs may be used to determine which chemicals are most likely to have endocrine disrupting effects is being further explored, and some progress has been reported recently notably with regard to AhR, ER, PXR and AR (e.g. Jacobs, 2004; Vedani et al 2005). A question also to be answered is how they could be used in conjunction with (Q)SARs that aim at predicting metabolism of chemicals.

The main *in vitro* tests for endocrine disruption comprise subcellular hormone receptor ligand binding, the induction of proliferation (mitogenesis) in hormone-responsive mammalian cell lines, and transactivation systems in yeast and mammalian cell lines. While most of the available tests at present are related to ER or androgen receptor (AR) activation, the question whether other hormone receptors also need to be examined can be raised. Therefore an ideal test that combines assessment of metabolism and of hormonal action should be easily adaptable to new endocrine targets that may become relevant and available for testing in the future.

Many subcellular, *in vitro* and animal tests have been listed in a compendium of methods for detecting EDs (Anon, 1996a), and such methods have been the subject of several reviews (see for some examples Table 1; see further Reel *et al.*, 1996; Holmes *et al.*, 1998; Eertmans *et al.*, 2003). An extensive evaluation and discussion of ED tests *in vitro* is presented elsewhere (NICEATM 2002a,b,c,d; ICCVAM 2003). Prevalidation of estrogen-binding, androgen-binding, estrogen and anti-estrogen transcriptional tests, androgen and anti-androgenic transcriptional tests is also underway at ECVAM. At present, several *in-vitro* assays that aim to detect endocrine disruptor activity are being considered at the level of the OECD. These include receptor binding assays based on GST-fused human ER, ligand binding domain expressed in *E. Coli*, MBP-fused human androgen receptor ligand binding domain expressed in *E. Coli*, rat uterine cytosol ER, rat prostate cytosol androgen receptors,

PanVera hrER α and PanVera rAR. Transcriptional activation assays with human or rat ER of both subtypes and human AR are also being investigated. Some of them exhibit stable receptor expression while others exhibit transient receptor expression. Examples are an MDA-kb2 cell line expressing AR, BG-1 cells stably transfected with estrogen-responsive luciferase reporter gene that express human ER, three hrER α binding assays (PanVera, Jena Biosciences and CERI) and ER-Calux, MELN, AR-Calux and PALM based on two human breast cancer cell lines, a bone cell line and a prostate adenocarcinoma cell line respectively. In order to better standardise the tests on the one hand and to make use of the possible intrinsic metabolism in these cell lines on the other, knowledge of the intrinsic metabolic capacity present in the cell lines considered for validation will be useful. Perhaps the presence of intrinsic metabolism may be one of the contributory factors for the ultimate test selection for the validation stage.

1.4.1 *In vitro* receptor binding assays

These assays provide a relatively rapid means of quantifying the ability of chemicals to bind to the ERs and AR particularly, and to competitively inhibit the binding of standard ligand (see Häggblad *et al.*, 1995). Theoretically they should even allow high throughput screening technology to be used, but this remains to be determined certainly if one would want to combine it with tests for metabolism. With regard to the ERs, the subtype that is being investigated is mostly the α -subtype. The assays involve assessing the molecular binding of the hormone to isolated and purified receptor protein. Competitive ligand binding assays, whether alone or combined with reporter gene expression, are designed to detect chemicals that directly interact with the NRs (Gray *et al.*, 1997). A further question that needs addressing is whether one (e.g. ER β) of the other subtypes of ERs should be incorporated in a test scheme. The AR and thyroid receptors have been proposed for inclusion into the screening for endocrine disruptors by EDSTAC. Mere ligand binding interaction studies cannot distinguish between agonists and antagonists and therefore it is imperative to have a functional *in vitro* test available that could do so. If metabolism is included in functional tests, one should realize that the agonist that will be used to obtain the background activation, against which an antagonist effect of chemicals can be determined, may also be

metabolised by the added metabolic enzymes. The development of functional tests for antagonism in the presence of metabolic competence of any kind will therefore require careful consideration of this problem. Substances like genistein and the medicinal products tamoxifen and raloxifene can behave as agonists, partial agonists or antagonists (Kauffman et al. 1997, Jordan and Morrow 1999, Dutertre and Smith 2000, Liu et al. 2003) depending on species, tissue and receptor examined. It would also be advisable to assure that the effects and nature of the binding interactions of such substances are precisely predicted by the tests to be developed.

1.4.2 Induction of hormone-responsive mammalian cell proliferation

Tests that are based on the induction of proliferation in estrogen-responding cells, particularly in the MCF-7 human breast cancer cell line, can be used to detect estrogenic activity (Fang et al. 2000; Ramamoorthy et al. 1997; Soto and Sonnenschein 2001). A sensitive reporter gene that uses green fluorescent protein expression, expressed in MCF-7 cells, was also used to detect compounds with estrogenic activity (Miller et al. 2000). Metabolism may have an impact on the effect of endocrine disruptors since some of them may stimulate the formation of 16 α -hydroxyestrone from 17 β -estradiol in MCF-7 cells (Miyamoto and Klein 1998). While the former is still estrogenic, it is also capable of damaging DNA. Although sometimes questioned, it is often considered as a possible biomarker of breast cancer, particularly in conjunction with other hydroxylated metabolites (Castagnetta et al. 2002, Ursin et al. 2002, Alvarez-Vasquez et al. 2003, and Fowke et al. 2003). It may influence the cell cycle and proliferative responses of MCF-7 cells (Lewis et al. 2001, 2005). Such an effect would then be attributable to an effect on estrogen metabolism rather than mediated by metabolism of the xenobiotic per se, but mutual interference between metabolism of endogenous hormones and xenobiotics should not be excluded. Proliferation assays are not recommended by ICCVAM because cell proliferation can be mediated through pathways other than those involving transcriptional activation of estrogen responsive genes (ICCVAM 2003). On the other hand, this could have the advantage of detecting endocrine disrupting mechanisms that are not mediated by an interaction with the

receptor, and if the cell line used would have also intrinsic metabolic capacity, the potential advantages may outweigh the possible shortcomings.

1.4.3 Transactivation assays (hormone-sensitive transcription of reporter genes)

These tests are based on the development of genetically engineered mammalian cells or strains of yeast (*Saccharomyces cerevisiae*) (Balaguer *et al.* 1996; Fang *et al.* 2000; Ramamoorthy *et al.* 1997). The cells have been transformed (or transfected) by introducing vectors bearing DNA sequences for human or mammalian ER NRs, in conjunction with the respective responsive elements linked to promoter regions for a reporter gene, together with the reporter gene itself. The transfected NR can function as a steroid-dependent, transcriptional activator. The induction of transcription of the reporter gene can be readily detected and quantified, (for example, colorimetrically, by using a suitable substrate [Joyeux *et al.*, 1997]). Receptor activation by a test chemical is detected as a stimulation of reporter gene expression following interaction with the response element. Reporter genes can be introduced into cells just for the duration of the experiment (transient transfection), or permanently, by developing a genetically altered and new cell-line (stable transfection). Yeast-based assays are not recommended by ICCVAM because many substances are poorly transported through the yeast cell wall (ICCVAM 2003). However a strain of genetically modified yeast (*Saccharomyces cerevisiae*) cells have been modified with respect to deletion of drug resistance transporters to overcome this problem (Sievernich *et al.* 2004), and are currently being developed further for receptors and P450 enzymes in addition to hER α (Lichtenberg-Fraté personal communication). It has also been suggested that another disadvantage of yeast-based assays is that they may not allow differentiating between agonists and antagonists (Dudley *et al.* 2000; Kohno *et al.* 1994, Legler *et al.* 2002b; ICCVAM 2003). Again, this problem can be overcome, this time by adaptations of plate designs (Kamp *et al.* 2005), and on the whole the data generated from a relatively low cost and easy to use system, does appear to be highly consistent with literature data. Use of the yeast estrogen screen as part of a testing battery may therefore be of value. Mammalian transactivation systems involve the use of luciferase, GFP, galactosidase or CAT reporter genes. For instance, Rogers and Denison (2000) have developed a stably transfected human ovarian cell line (BG1), using the luciferase gene as a reporter gene, for the detection of

estrogenic and anti-estrogenic chemicals. Similarly 293 HEK cells can be transfected with ER β receptors (Lemmen et al. 2002; Meerts et al. 2001). Transactivation assays have also been generated that make use of endogenous receptors, such as the human breast cancer cell line-derived MVLN (Demirpence et al., 1992; Freyberger and Schmuck 2005) and ER CALUX (Legler et al, 1999) assay, using MCF7 and T47D cells, respectively. Interestingly, ER CALUX cells have been used in combination with S9 (Legler et al. 2002a, b).

2. Metabolism

2.1 The role of metabolism in the activation and detoxification of xenobiotics: Phase I and Phase II metabolism

Information on the metabolism of a substance is important in the evaluation of its toxic potential. For example, the determination of metabolic stability can provide information on the potential for bioaccumulation and whether a chemical is likely to be converted to a more active form (metabolic activation) or a less active form (metabolic detoxification). With regard to endocrine disruptors, one should take into account that metabolism of the xenobiotic may not only alter its own potency by creating metabolites that are either more or less active than the parent compound. Hormones and in particular steroid hormones are formed or metabolised by enzymes that participate in the metabolism of xenobiotics. Prominent examples of such enzymes are the cytochromes P450 (CYPs). A chemical that is metabolised by these enzymes may, by mere interaction with the enzyme, act as a competitor for the hormone or hormone precursor and therefore disturb hormone levels and hormonal equilibrium.

A substance that is absorbed orally from the gastrointestinal tract is transported via the portal circulation to the liver, where it may be subjected to hepatic metabolism, followed by elimination in the bile or via the kidneys. There is also the possibility of extra-hepatic metabolism.

A typical chemical metabolism pathway involves the oxidation of the parent substance (phase I oxidation), followed by conjugation of the oxidised moiety with highly polar

molecules, such as glucose, sulphate, methionine, cysteine, glutathione or glucuronic acid (phase II conjugation). The key enzymes for phase I oxidation are the isoforms of the CYP family of enzymes. The superfamily of CYPs are heme-containing enzymes with very wide substrate specificities by virtue of their existence in a large number of isoforms or isozymes. CYP enzymes can often be induced or inhibited by exogenous chemicals that may exert endocrine effects through this mechanism. While the liver is the main site of xenobiotic metabolism, phase I and II metabolism also occurs in most tissues including the gut microflora (Combes, 1992, Coecke et al., 2006).

Interactions between different EDs are generally considered a major issue. One possibility for EDs to interact with each other may be at the level of enzyme induction or inhibition as is often the reason for drug-drug interactions (Lin and Lu 2001). Enzyme induction or inhibition is not only a possible means for chemicals to exert endocrine disruption but it can ultimately also alter the metabolism of the chemical itself. Inhibition of CYPs (and thus risk for interactions) can be studied using incubations with microsomes or cell lines in combination with substrates specific for CYP isozymes or phase II enzymes in the absence and presence of EDs (Brandon et al. 2003, Tucker et al. 2001). By measuring the effect on the disappearance of the substrate, the inhibition capacity of the ED can be determined. The induction of CYPs by EDs can be investigated by measuring the binding to nuclear receptors constitutive androstane receptor (CAR) and pregnane X receptor (PXR) or the CYP mRNA levels (Xu et al. 2005, You 2004, Sparfel et al. 2003, Sonoda et al. 2003, Friedman et al. 2002, Synold et al. 2001). Figure 1 indicates the principal NRs that induce key P450 transcription factors for phase I metabolism of steroidal, xenobiotic and endocrine disrupting substances.

Furthermore, the metabolism rate of a model substrate in cell lines pretreated with EDs can be determined (about 48 h is necessary for CYP induction) (Brandon et al. 2003, Tucker et al. 2001). Genetics (such as CYP polymorphisms), disease, diet, cigarette smoking, age and environmental factors may determine the induction or inhibition of CYPs (Lin and Lu 2001). This may mean that certain subgroups may be more susceptible than the general population.

CYPs catalyse mixed function oxidase activities by introducing oxygen into a molecule, thereby rendering it more polar. The major human CYPs involved in chemical metabolism are CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP3A5, CYP3A7 and CYP4A11 (Lewis, 1996; Clark, 1998; Soucek, 1999; Masimirembwa *et al.*, 1999; Ozawa *et al.*, 2000; Tanaka *et al.*, 2000; Riley *et al.*, 2001; Sheweita, 2001; Nishimura *et al.*, 2003; Furukawa *et al.*, 2004; Lanza *et al.*, 1999; Sheets *et al.*, 2004; Ding and Kaminsky, 2003). Some of these are mainly active in the fetal liver and not detectable in adults (see section 2.5). Of the different CYP enzymes, CYP3A4 is the most abundant in the human liver, and plays a key role in the metabolism of steroids and xenobiotics: metabolising more than 50% of all currently prescribed drugs. Polymorphisms and interindividual variability in the expression of these enzymes are a key reason why different individuals react differently to chemicals, and also a major reason for the wide range of experimental variability with tests based on human liver tissue (Lin and Lu 2001).

The key phase II enzymes include N-acetyl transferases (NAT), UDP-dependent glucuronosyl transferase (UGT), sulphotransferases (SULT), sulphatases, and glutathione S-transferases (GST). The structures and functions of phase II enzymes have been reviewed elsewhere (Burchell *et al.*, 1998; Glatt, 2000; MacGregor *et al.*, 2001; Turan *et al.* 2005). These enzymes catalyse the conjugation of the metabolites generated by phase I metabolism to water soluble moieties such as glucuronic acid and glutathione, which traps electrophilic intermediates. However, although phase II metabolism is generally considered to be detoxifying, it can also generate powerful electrophiles that are highly reactive (Combes, 1992). The phase II enzymes can also conjugate certain groups in the parent compound without precedent phase I metabolism. This has been reported for instance for aromatic hydroxyl groups in beta-adrenergic agonists (Smith 1998) or for halogenated compounds (reviewed by Guengerich 2005).

The sulphation of estrogens and thyroid hormones by SULT enzymes and sulphatases respectively is a particularly important route for the removal of active hormones from the body. In mammals SULT enzymes create a circulating pool of estrone sulfate that can be

converted back to active estrogens in peripheral tissues. With respect to estrogen metabolism and ED impacts, ED chemicals such as bisphenol A, alkylphenols and phthalate plasticizer, as well as plant phytoestrogens, have been shown to inhibit the inactivation of estrogens via the major SULT isoforms: estrogen sulphotransferase (SULT1E) and phenol sulphotransferase (SULT1A1) (Nishiyama et al 2002, Kirk et al. 2003; Waring and Harris 2005). As part of the series of European Commission funded research programmes, *in vitro* assays utilizing both primary human liver and fish liver tissue, human neuronal (SK-N-SH), gastrointestinal (HT-29) and medulloblastoma (TE 671) cell lines, all of which express SULT isoforms, have been developed for screening SULT inhibition by Waring's group, (Davies et al 2004; Harris et al. 2005; Kirk et al 2003; Turan et al., 2005). These known indirect or non-genomic mechanisms indicate that the phase II metabolism may be of particular importance and must be investigated thoroughly in the context of *in vitro* ED testing.

2.2 *In vitro* methods for the measurement of metabolism

2.2.1 *Current status of computer-based approaches for assessing metabolism*

A number of computer-based expert systems for predicting metabolism and metabolism-dependent toxicity are undergoing development, and have been reviewed elsewhere (Dearden et al. 1995; Cronin, 2001).

An example is the METEOR system, a rule-based system for the prediction of metabolism which is being developed by LHASA (Greene et al., 1999). METEOR consists of a knowledge base of biotransformations that describe the metabolic reactions catalysed by specific enzymes, and that are related to specific substrates. The system can be linked with DEREK, to provide a means of assessing both metabolism and toxicity.

Two other rule-based expert systems, HazardExpert and MetabolExpert, are being developed and marketed by CompuDrug Chemistry Ltd. HazardExpert enables predictions to be made for a number of toxicological endpoints, taking into account factors such as species, dose, route and exposure duration. HazardExpert can be linked to MetabolExpert, which makes

qualitative predictions of the metabolites of compounds. These predictions are made by using a rulebase consisting of molecular fragments. By linking HazardExpert with MetabolExpert, the user can obtain predictions of toxicity not only for the parent molecule, but also for the potential metabolites of the molecule.

Recently, a new rule based approach has been developed where predicting the role of metabolism in selective toxicity and adverse health outcomes was associated with a probabilistic framework. Specific metabolism simulators have been developed for predicting biodegradation. CATABOL (Jaworska et al., 2002) is a tissue metabolism simulator. TIMES (Mekenyan et al., 2004a) uses a heuristic algorithm to generate plausible metabolic maps from a comprehensive library of biotransformations and abiotic reactions and estimates system-specific transformation probabilities. The transformation probabilities have been calibrated to specific reference conditions using transformation rate information from systematic testing or databases with observed (thermodynamically stable) metabolic products. The quantitative evaluation of transformation by their probabilities (plausibility estimates) allowed prioritisation of metabolites by their stability, reactivity, solubility, etc. The simulators have been combined with toxicity predicting tools allowing predicting metabolic activation of chemicals in integrated systems; thus, the TIMES system has been used to predict mutagenicity and skin sensitisation accounting for metabolism of chemicals (Mekenyan et al., 2004a; 2004b)

Another approach to the prediction of metabolism (and metabolism-dependent toxicity) is provided by the Computer-Optimised Molecular Parametric Analysis of Chemical Toxicity (COMPACT) method of Lewis and coworkers (Lewis et al., 1998). This method can be used to predict whether a molecule has the potential to act as a substrate for one or more of the CYPs, or the ability to promote peroxisome proliferation.

The COMPACT method is based upon the premise that there are certain structural requirements of a molecule that make it susceptible to oxidative metabolism. Firstly, molecules must be capable of binding to CYPs, due to their planar molecular shapes.

Secondly, molecules that are capable of binding to a CYP must also be susceptible to chemical oxidation. The COMPACT approach is therefore based on two physicochemical descriptors, molecular planarity and electronic activation energy. Molecular planarity is a function of the cross-sectional area and molecular depth of the potential substrate, whereas the electronic activation energy is the difference between the energies of the highest occupied and lowest unoccupied molecular orbitals. A two-dimensional plot of molecular planarity and electronic activation energy for a series of molecules reveals that they can be divided into categories according to the particular CYP by which they are metabolised (Parke et al., 1990).

The prediction rate obtained with the COMPACT system was found to be improved when its predictions were considered in combination with those generated by the HazardExpert system (Brown et al., 1994), demonstrating the usefulness of a battery approach to prediction. The COMPACT approach has been extended to include the molecular (protein) modelling of the CYP enzymes themselves (Lewis et al., 1999) and NRs (e.g. Jacobs et al 2003). The use and potential of crystal structures and molecular modelling of NRs and CYPs, for validation as *in silico* alternative tools for hazard assessment, were examined more closely at an expert ECVAM Workshop on Molecular Modelling Approaches for Human Hazard Assessment of Chemicals in February 2006 (Jacobs et al., 2006 workshop report in preparation).

Another computational method for predicting potential metabolites is the META system, which is part of a suite of programs developed by Klopman and colleagues (Klopman et al., 1994; Talafous et al., 1994). The rules in the META rulebase were taken from the biochemical literature, rather than derived by a statistical approach.

The large diversity of chemicals that might interfere with hormone function and the many potential molecular targets may make the establishment of (Q)SARs for EDs and for the potential to be metabolised or to affect metabolism of endogenous hormones or ED's difficult but the possibility should and is being explored further. For instance the US EPA

have recently initiated an international chemical prioritization research project (ToxCast) that aims to evaluate chemical properties and effects, including ED effects across a wide range of information domains.

2.2.2 Types of metabolising systems applied to *in vitro* test systems

A number of *in vitro* systems are available for studying metabolism. These include: precision-cut tissue slices, subcellular fractions such as the microsomal fraction, primary cells in suspension, primary monolayers of cells in culture, continuous cell lines, immortalized primary cells, liver-derived cell lines re-expressing biotransformation enzymes and genetically-engineered cell lines expressing biotransformation enzymes (Schmalix et al., 1996; Clarke, 1998; Philip et al., 1999; Coecke et al., 1999, 2000; Bull et al., 2001). These *in vitro* tests can incorporate important toxicological endpoints, including cytotoxicity and genotoxicity. It would be worthwhile to investigate whether endocrine endpoints could also be expressed in these cell lines or whether proper indicator cells can be co-cultured in cell lines with metabolic capacity. The precision-cut tissue slices may be less useful from the technical point of view if they need to be combined with other cells or subcellular fractions, as indicators of effect, as will be required for simultaneous testing of metabolism and ED effects.

To date, no *in vitro* methods for determining phase I or phase II biotransformation, or for evaluating metabolism-dependent toxicity, have been validated according to ECVAM's principles and procedures. The current OECD Test Guideline 417 for assessing the toxicokinetic effects of chemicals (OECD, 1984) is based on *in vivo* studies. However, many studies provide support for the usefulness of *in vitro* methods for assessing metabolism and metabolism-dependent toxicity. From such studies, it is evident that there are enormous species differences in toxicokinetics; this is especially true for the metabolic differences between humans and rodents (Ozawa et al., 2000). Therefore, there is a great need for human-based *in vitro* models that would offer better predictions of potential hazard to humans than could ever be obtained from laboratory animal studies (Coecke et al., 1999, 2000, 2001; Doehmer et al., 1999). The status of the currently available approaches for assessing metabolism is summarised in Table 2.

Recently, there has been success in relating the rate of *in vitro* metabolism of several compounds with the corresponding events *in vivo*. This represents a major step, since the value of *in vitro* metabolism systems has traditionally been considered to be purely qualitative in nature. The basis of this relationship is the use of the parameter, intrinsic clearance, which is a pure measure of enzyme activity toward a chemical *in vivo* and is not influenced by other physiological determinants of clearance, such as hepatic blood flow or drug binding. The *in vitro* equivalent of this parameter is the $V_{max}:K_m$ ratio. The utility of *in vitro* intrinsic clearance as a predictor of *in vivo* intrinsic clearance, and therefore hepatic clearance and total body clearance, has been assessed with a database of 35 drug substrates for rat CYPs (Houston & Carlile, 1997). Other studies have demonstrated the usefulness of the intrinsic clearance approach for predicting kinetics in humans (Obach et al., 1997; Ito et al., 1998).

Subcellular systems are added to *in vitro* test systems as enzyme homogenates. Such homogenates can comprise:

- purified enzymes
- cytosolic soluble enzyme fractions (S100 supernatant)
- microsomal particulate enzyme fractions (S100 pellet)
- post-mitochondrial S9 supernatant (consisting of both cytosol and microsomes).

All of these systems are capable of activating and/or detoxifying xenobiotics.

In addition, the prostaglandin H synthase (PHS) system has been added exogenously to *in vitro* toxicity assays (Combes, 1992). This enzyme catalyses the first two steps in the synthesis of prostaglandin, thromboxane and prostacyclin. Two forms of the enzyme have been characterised (PHS 1 and 2) and both are heme proteins that exhibit both cyclooxygenase and peroxidase activity, the latter being responsible for the one electron oxidation of a wide range of xenobiotics (Eling *et al.*, 1990). The peroxidase activity of PHS can be activated *in vitro* if enzyme extracts are supplemented with arachidonic acid, and several chemicals are activated by the PHS system to mutagens and carcinogens (Woo *et al.*,

1988; Flammang *et al.*, 1989). Microsomes from ram seminal vesicles have been shown to have a high level of PHS activity.

Cellular enzyme systems can also be used in *in vitro* assays, and these fall into one of three main categories: a) residual metabolism of the indicator cells; b) the use of co-culture systems comprising the indicator cells and a metabolically-competent cell line (e.g. freshly isolated hepatocytes or genetically-engineered cell lines); and c) the use of genetically-engineered cell lines that simultaneously act as both indicators of toxicity and sources of metabolism.

The contribution of the indicator cells themselves to metabolism, with or without the addition of exogenous metabolism is unavoidable and should not be overlooked when interpreting toxicity data (Combes, 1992). In the case of residual metabolism, often the level and specificity is unknown and although it is usually assumed to be low its presence can complicate the situation. To standardise the detection system to the largest possible extent it seems imperative to study the intrinsic metabolism in the indicator cells that will be used in the future. It could even be part of a validation effort to describe the intrinsic metabolic capacity in the indicator cells. Only when co-culture systems or enzyme fractions would be used, the residual metabolism in the target cell type may become less important because it would be overwhelmed by the added metabolic capacity. If the residual metabolising capacity of indicator cell lines would be sufficient to produce active metabolites of inactive parent compounds and to deactivate EDs, this might constitute an unexpected but useful model to study metabolism and an ED mechanism simultaneously. If such models would indeed exist, a next step to further improve them may be to enhance the metabolic activity, to make it more reproducible and predictable and finally to make the level of metabolic activity a better simulation of the *in vivo* situation by using genetic engineering techniques. This possibility is another reason why residual metabolism in all cell lines that will be used in validation studies for ED testing should be determined.

The advantage of exogenous metabolising systems is that the enzymes are in direct contact with the xenobiotics without the need to first penetrate into the cell. However, unless the toxicity system is subcellular (e.g. a receptor binding assay) or the target is located in the cell membrane (e.g. membrane receptor), the resulting metabolites have to enter the cells to exert their effect. As a major target for EDs are NRs, penetration of the formed metabolite and/or the parent compound into the cell will be required in most cases. In the case of co-culture systems, the xenobiotics have to enter the metabolically-competent cells, then leave them and enter the indicator cells or interact with targets located in their membranes. In cells genetically-engineered to metabolise and detect toxicity, the metabolites are generated in close proximity to the target site(s) for toxicity if they are located intracellularly but they need to exit the cells again if they are acting upon membrane bound molecular targets. As receptors for hormones such as estradiol may be located intracellularly or on the membrane, the location of the target and site of metabolite formation may be important issues to consider with regard to models intended for study of the metabolism and effects of EDs. The transport through the cell may need particular attention if transport proteins are present in the *in vitro* system utilised.

There are many different cell lines that have been genetically-engineered to express various phase I and phase II enzymes and also a combination of both for use in toxicity testing (Doehmer, 1993; Macé *et al.*, 1998). In a review by Friedberg *et al.* (1999), a comparison was made of insect, bacterial, yeast, and mammalian metabolism models. In general, the catalytic properties of CYPs in the various models were rather similar.

A recent and promising innovation is the use of established cell lines, such as V79 cells, which have been genetically engineered to express selected genetic variants of human CYP enzymes, so that polymorphic effects can be assessed (Coecke *et al.*, 2001). Very recently, a V79 cell line has been generated that expresses mouse CYP 2E1 to permit interspecies comparisons (Bernauer *et al.*, 2003b). These cell lines, in common with several others, have a number of benefits including a stable diploid karyotype, no CYP background activity, and they can be transfected with rodent and human CYPs. This allows an investigation of the

contribution of specific isozymes to metabolism and also studies on species specificity. However at a recent ECVAM workshop on *in vitro* models to study metabolism, there was consensus that at least at present, only limited numbers of P450 can be expressed in the same cells without affecting their viability (Coecke et al 2006).

A further consideration when choosing a metabolising system is the well-known problem of preferential activation *in vitro*. This is due to the fact that the use of enzyme homogenates under normal conditions favours Phase I metabolism, rather than conjugation reactions. This can be overcome to some extent by adding cofactors for phase II reactions, but this is usually taken better into account when using whole cells such as hepatocytes, and others that express both phase I and II reactions. At the recent ECVAM workshop, the use of human hepatocytes was not considered to be possible at present for screening on a large scale.

2.3 The relevance of metabolism to the testing of endocrine disruptors

One of the most frequently cited limitations of *in vitro* assays concerns the qualitative and quantitative deficiencies in the metabolism of test chemicals, in comparison with *in vivo* metabolism. This is a particularly important consideration when testing EDs since several, hormonally-active chemicals, including some naturally-occurring EDs, are known to be subject to metabolism (Table 3). False positive data could be generated due to the lack of metabolic detoxification of test chemicals, and false negative data could arise, if the required metabolic processes for activation are lacking in the *in vitro* system used.

Most information on metabolism of hormonally active substances has been derived principally from studies with endogenous steroids, but of course it applies in principle equally to exogenous steroids with potential ED activity, and to any chemical that can modulate the homeostasis of the endocrine system (Honkakoski and Negishi, 2000). In addition, the effects of certain EDs are due to interference with the metabolism of the endogenous hormone and hence with its biologically available concentration. In other words, the use of test systems capable of metabolism should be an integral part of ED testing. The need to model metabolism is an argument that has been used in part to justify the need for

animal-based tests for EDs, but it is an equally important reason to incorporate metabolic capacity in *in vitro* assays.

Methoxychlor (bis[p-methoxydichlorophenyl]trichloroethane - MC) has estrogenic activity *in vivo* in a mouse uterotrophic assay, while it is inactive or virtually inactive *in vitro* in mouse uterine ER binding and HeLa ER cell transcriptional activation assays (Shelby et al. 1996). Substances like the di-hydroxylated metabolite of methoxychlor, 2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE), are estrogenic and anti-androgenic *in vitro* and their formation *in vivo* can explain the estrogenicity and antiandrogenicity of methoxychlor (Gaido et al. 1999). Methoxychlor is an interesting example of an ED that is metabolised because it illustrates many of the aspects related to metabolism that are important for the action of endocrine disrupters and that need to be taken into account when developing tests that will measure metabolism and endocrine effects *in vitro*. This chemical undergoes sequential de-methylation by CYPs 2C19 and 1A2 yielding mono-demethylated and bis-demethylated metabolites. These metabolites are hydroxylated at the ortho position on the ring to yield phenolic groups by CYPs 3A4 and 2B (Stresser and Kupfer, 1998a 1998b; Dehal and Kupfer, 1999). CYP2C19 converts methoxychlor into a phenolic monodemethylated derivative that can be further hydroxylated in the ortho position by CYP3A4 while CYP3A4 does not cause O-demethylation or hydroxylation of methoxychlor (Stresser and Kupfer, 1998b). This observation means that a prosubstrate is turned into a substrate by one CYP for another CYP (Stresser and Kupfer, 1998b). In other words caution is needed before concluding that a particular P450 is not involved in the metabolism of a compound based on the use of an individual cDNA-expressed enzyme (Stresser and Kupfer, 1998b). CYPs known at present to be involved in the metabolism of methoxychlor are CYP1A2, 2A6, 2C8, 2C9, 2C19 and 2D6 for O-demethylation, CYP3A4, 3A5 and (rat) 2B1 for ortho-hydroxylation, CYP 2B6 for O-demethylation and ortho-hydroxylation (Hu and Kupfer, 2000a; 2000b). CYP2B6 has, however, a very narrow substrate specificity (Hu and Kupfer, 2000b).

The bis-O-dimethylated metabolite of methoxychlor HPTE has estrogenic activity and is more potent and has higher efficacy than the parent compound (Bulger et al. 1978; Beresford et al., 2000; Shelby et al., 1996). HPTE also inhibited seminiferous cord formation *in vitro* much more than the parent compound methoxychlor (Cupp and Skinner 2001), probably again demonstrating its more pronounced estrogenic effect. HPTE also depresses gonadotropin-releasing hormone expression in a murine neuronal cell line probably because it is an estrogenic agonist (Roy et al., 1999). In another study, it was demonstrated that bis-OH-methoxychlor is a more potent estrogen agonist than mono-OH-methoxychlor that in turn is more potent than methoxychlor (Kupfer and Bulger 1987). Moreover methoxychlor can also be converted into the metabolite 1,1-dichloro-2,2-bis(4-methoxyphenyl)ethene which is also a proestrogen. The bis- and mono-hydroxylated metabolites of this product are also estrogens (Kupfer and Bulger 1987). These authors have used rat liver microsomes and NADPH as the metabolising system in the presence of immature rat uteri ER as the bioassay. If this test system could be standardized, validated and preferably make use of human microsomes, it may be one way of incorporating metabolism by human enzymes into *in vitro* testing for endocrine disruption.

HPTE has anti-androgenic effects because it is a receptor antagonist (Maness et al. 1998). In addition, HPTE also decreased the production of testosterone by rat Leydig cells because it reduced steady-state messenger ribonucleic acid levels of the CYP cholesterol side-chain cleavage enzyme (Akingbemi et al., 2000). This means that the compound may have anti-androgenic effects by decreasing the amounts of natural agonist that are available. In addition, methoxychlor and its bis and tris hydroxylated metabolites inhibited several other CYPs (Li et al. 1993).

The estrogenic potency of methoxychlor in the yeast cell assay was markedly increased after incubation with human hepatic microsomes because of the conversion to metabolites including HPTE (Elsby et al., 2001a). Although the yeast cell assay is frequently used and it might be a good candidate to be used in conjunction with human microsomes if sufficiently standardized and validated, as previously noted there can be potential shortcomings that

have led to some objections against its use (ICCVAM 2003). In another *in vitro* system employing a recombinant yeast cell bioassay expressing the human ER α linked to a reporter gene, the estrogenic effect of the potential methoxychlor metabolite bishydroxymethoxychlor was larger than that of the parent compound (Coldham et al., 2002). The activity of potential metabolites of mestranol and isoxanthohumol (17 α -ethynyl estradiol and 8-prenylnaringenin respectively) at the ER α was also larger than that of their parent compounds (Coldham et al. 2002). Incubation of mestranol or methoxychlor with rat liver microsomes that were activated with Aroclor 1254 (S9 fraction), increased the potency of these compounds in the bioassay. Metabolites that were identified and possibly responsible for the increased activity, included 17 α -ethynyl estradiol for mestranol and monohydroxy- and bishydroxy-methoxychlor for methoxychlor (Coldham et al. 2002). There was no evidence for metabolism of isoxanthohumol in this combined assay (Coldham et al. 2002). Mestranol was also activated by microsomes that were induced with saline (control), β -naphthoflavone, 3-methylcholanthrene, isoniazid, pregnenolone-16 α -carbonitrile but not phenobarbitone (Coldham et al., 2002).

It should be noted that HPTE is a preferential ER α agonist with antagonist activity on ER β and the AR in human hepatoma cells (Gaido et al., 1999; 2000) and that the gene expression that is caused by HPTE in mice is similar but not the same as that caused by estradiol (Waters et al., 2001). The pattern of activity on ER α , ER β and ARs in human hepatoma cells may be different among close analogs of methoxychlor (Gaido et al. 2000). These findings further illustrate that functional assays are needed when including metabolic capacity into ED screening and they also illustrate that it may be necessary to study different subtypes of ERs.

As an androgen antagonist, HPTE is about 10 times more potent than methoxychlor in a HepG2 human hepatoma cell line transiently transfected with the human AR and an androgen-responsive reporter (Maness et al, 1998). This suggests that under these particular assay conditions, the cells did not have the metabolic capacity to convert methoxychlor into its metabolite. This raises the question whether hepatocytes with metabolic competency and

containing the SHRs mentioned can be constructed to produce a more useful screening assay.

The bis- and trishydroxymethoxychlor and a catechol methoxychlor metabolites can be formed from methoxychlor *in vitro*, by incubation with supersomes that have human cDNA expressed CYPs or when incubated with human liver microsomes (Hu and Kupfer, 2002a). The metabolism of methoxychlor by various CYPs, under these circumstances, is even subject to specificity towards the enantiomers (Hu and Kupfer, 2002b).

Another study showed that Aroclor-1254 induced rat liver S9 fractions are compatible with MCF-7 estrogenic assays and that the activity of methoxychlor was increased while that of estradiol was decreased by co-incubation of the assay cells with S9 (Charles et al. 2000). This illustrates that the combination of S9 systems with MCF-7 cells is technically possible and the system is able to detect estrogenic activity of compounds that need to be converted to an active metabolite.

Methoxychlor metabolites interact with the CAR and induce CYP2B via a mechanism unrelated to estrogenic activity (Blizard et al., 2001). It is known that induction of enzymes including many CYPs and phase II metabolism enzymes can occur through NRs and in particular the CAR, the PXR (Lehmann et al 1998; Handschin and Meyer 2003) but also the glucocorticoid receptor and the peroxisome proliferator-activated receptor α (Hines et al. 2001, reviewed in Jacobs 2005). CAR and PXR are responsible for the induction of various CYPs (CYP2B and CYP3A respectively) and drug transporters in human cells, but they do not influence the basal expression levels (Friedman et al. 2002). Phenobarbital and (1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) are known agonists of CAR, whereas lipopolysaccharide is an inhibitor (Wei et al. 2000, Tzamei et al. 2000). PXR activation leads to induction of CYP3A, but also to a lesser extent CYP2B and there are extensive species differences in xenobiotic PXR activation. Dexamethasone and rifampicin are known agonists of human PXR (Moore et al 2000a; Friedman et al. 2002; Frank et al. 2005;) as are some phytochemicals and their metabolites, organochlorine pesticides and bacteriocides

(Moore et al 2000b; Jacobs et al 2004). The anticancer drug ET-743 is an inhibitor of PXR (Sparfel et al. 2003, Friedman et al. 2002). The effect of a potential ED can be investigated in several hepatic and kidney cell lines the Hep G2 cell line by measuring the CAR and PXR levels in the absence or presence of the substance and also in combination with a known agonist of CAR or PXR respectively (Goodwin et al. 2002, Friedman et al. 2002). Furthermore, CYP2B6 and CYP3A4 mRNA levels can also be used to investigate the effect on CAR and PXR (Friedman et al. 2002). The cells should be incubated with an ED alone or in the presence with a known inducer of CAR or PXR. If the ED leads to induction of CYP mRNA, the effect on the receptor should be verified with a known inhibitor of CAR or PXR. The mRNA levels can be measured with real-time reverse transcriptase-polymerase chain reaction (RT-PCR) with specific primers for CYP2B6, CYP3A4, CAR and PXR and a reference gene with stable expression in the cell (e.g. GAPDH). Whether a ligand-binding assay for these receptors or the measurement of mRNA for the enzymes involved in metabolism, might be a valuable tool to predict induction of metabolism by a number of chemicals might depend upon the number of positives in such tests, the ultimate value for the prediction of their *in vivo* effects and the additional costing.

Western blotting and PCR may provide a very good methodological tool to measure mRNA levels of certain CYP enzymes in order to evaluate the effects of a certain chemical on a certain receptor. Nevertheless, measurement of mRNA does not necessarily reflect the induction of xenobiotic metabolising CYP enzymes at the protein or activity level (Sumida et al., 1999; Tew et al., 1996). Therefore, the induction of certain xenobiotic-metabolising CYP enzymes could also be investigated in the following way (in the case of CYP2E1, this has been applied in Bernauer et al. 2003a, b): cells can be incubated with an inducer and with the probe substrate of the CYP enzyme to be investigated. After incubation, the metabolites (specifically formed under the influence of a certain CYP enzyme) can be quantified by well established activity assays (given in Table 5). Alternatively, cell protein can be isolated after culture. Cell protein can be incubated with the CYP substrate and metabolites can be quantified. In addition, Western Blotting analysis of the relevant CYP enzyme can be performed in the cell protein by using commercially available antibodies.

In rats exposed to methoxychlor for 4 days, a decreased conversion of thyroxine to triiodothyronine was observed, probably because one or more metabolites bind to 5'-monodeiodinase type 1 (Zhou et al., 1995; Morrell et al., 2000). It is not immediately clear whether this is an important effect for the endocrine actions of methoxychlor, nor whether it would occur with other compounds. This finding may reinforce the idea that considering testing for thyroxine metabolism and thyroid receptor activation may indeed be worthwhile at some stage of ED screening.

Methoxychlor and its metabolites, including mono- bis- and tri-hydroxylated compounds, may bind covalently to hepatic microsomal proteins, and this is catalysed by CYPs (Bulger and Kupfer, 1990), but demethylation is not an essential prerequisite. This finding is an illustration of the potential importance of protein binding in *in vitro* assays.

In catfish, methoxychlor alone or in combination with β -naphthoflavone alters the capacity of liver microsomes to metabolise methoxychlor *in vitro* (Schenk et al. 1997). Pretreatment with the CYP1A-inducer β -naphthoflavone did not affect the metabolite pattern of methoxychlor alone, but when administered simultaneously, a reduced metabolism of methoxychlor was observed and yet estrogenicity was increased (Schenk et al. 1997). While it is not clear in this paper which factor(s) caused the increased estrogenicity, the involvement of cross-talk between additional receptor and non-receptor mediated pathways, particularly with respect to the recently elucidated relationship between AhR and ER (Ohatake et al 2003), also PXR, and suppression of SULT enzymes (thereby increasing the pool of free estrogen) for example could be part of the explanation (reviewed in Jacobs 2005). An additional part of the explanation may also be species differences in the sensitivity for certain metabolites.

The example of methoxychlor indicates that metabolism can influence the endocrine disrupting properties of a chemical in different ways by forming metabolites with increased potency and efficacy, by forming metabolites that act on different molecular targets, or that have an increased potency at one receptor and that become antagonists at different receptors.

One should thus consider testing of metabolites for different endpoints, as part of a tiered testing strategy, to tease out the mechanistic basis underlying the key steps in interrelated metabolic pathways. It seems most useful to include one particular molecular target or effect in each test to obtain reproducible results that can be easily interpreted. On the other hand, the potential effects of metabolites on different targets and the possibility of one metabolite being an agonist on one system and an antagonist on another system may make the extrapolation of individual test results to the intact organism difficult but not impossible. If one considers metabolism of chemicals, the effect of different enzymes in cascade may be quite different from the sum of effects of individual enzymes, and metabolism may even not take place when using only one enzyme. It may thus be more realistic to have as many enzymes available as possible in one particular test in amounts relevant to the *in vivo* situation. This implies that several metabolising enzymes would need to be incorporated into cells if one uses genetically engineered cells for testing. One question to be answered is whether particular metabolites that may be more potent than the parent compound are formed in sufficient amounts to significantly contribute to the ultimate effect. Obviously the amount of the different enzymes expressed, and their activity in the test should therefore be representative for the situation in the intact organism. This may not be easy to achieve with genetically engineered cells designed to express the enzymes involved. A workshop that was recently organized by ECVAM also revealed that it is even not yet possible at this moment to express many different CYPs in the same cells and keep them in culture. For these reasons, the use of genetically engineered cells may be possible in the near future, and in the long run may represent the ideal test method. Maybe, as a first approximation, parallel or sequential use of different cell lines expressing different xenobiotic metabolising P450 enzymes might be discussed. On the other hand fully competent metabolising cells like hepatocytes are not yet useful for validation screening purposes (with respect to reproducibility) on a large scale, and in particular if human hepatocytes are required (see Blauboer B. et al. 1994). Availability and variability (different age, gender, condition and also loss of P450 expression/activity) will constitute a problem and it is not evident to grow hepatocytes in culture under stable conditions to allow screening of large series of chemicals. Furthermore, the reproducible expression of the molecular target that would

constitute the endpoint to be measured in the hepatocytes or viable co-cultures with the indicator cells would be required.

The finding that metabolism by certain cytochromes may be specific for some enantiomers and the likelihood that at least for a number of chemicals the interaction with the target receptor may also be specific for certain enantiomers may indicate that particular attention will need to be paid to the testing of chemicals with chiral centers.

Furthermore the interference by methoxychlor or its metabolites with the turnover of thyroid hormones suggests that the metabolism of other hormones than estrogens may need more consideration than it has received in the past.

Perhaps the most important findings with methoxychlor are that it seems possible to combine the use of S9 fractions or microsomes with *in vitro* assays that measure estrogen function, allowing detection of agonist and antagonist effects, in indicator cells that contain human ER α . Detection of activity from the metabolites that are formed is possible. The assays involved are the MCF-7 and the yeast cell assay that are often used and relatively well known. The latter test may be equally suited for incorporation of other hormone receptors, which would broaden the scope of its usefulness. The assay that was mentioned earlier as the ER CALUX (Legler et al. 2002a, b) also uses a combination of S9 and measurement of estrogenic activity. Provided that the test can be made sufficiently specific it may offer an alternative testing method (Rogers and Denison 2000).

Another example of the importance of metabolism in the determination of endocrine effects is the conversion of tamoxifen to its 4-OH derivative. This conversion is required to confer increased anti-estrogenic activity on the molecule (Cassidy and Milligan 1998) and is catalysed by CYPs 3A4 and 2D6 (Dehal and Kupfer 1998, 1999). The mandatory conversion of chlorobiphenyl to 2-chloro-4-hydroxy-biphenyl to become estrogenic (Rosenkranz, unpublished) further illustrates the need for incorporation of metabolic capacity in *in vitro* tests. Aryl methyl sulfone metabolites of polychlorinated biphenyls (PCBs) and of 2,2-bis(4-

chlorophenyl)-1,1-dichloroethene, on the other hand, can behave as estrogen antagonists (Letcher et al. 2002). Methyl sulfonyl PCBs also interact with glucocorticoid receptors (Johansson et al. 1998).

Dexamethasone is an example of a synthetic steroid with therapeutic activity, which is hydroxylated in a regio-selective way (Lewis, 1996; Amrani et al., 1999).

Polybrominated diphenyl ethers (PBDEs) and some of their metabolites exert *in vitro* estrogenic effects in three different cell line assays based on ER-dependent luciferase reporter gene expression (Meerts et al. 2001), but these findings have not been confirmed. The purity of PBDE (as with every substance) should be carefully assessed before use in an assay.

Vinclozolin forms two metabolites: 2-[[3,5-dichlorophenyl-carbamoyl]oxy]-2-methyl-3-butenic acid, also termed M1, and 3',5'-dichloro-2-hydroxy-2-methylbut-3-enamide, also termed M2 (Wong et al. 1995). While both are AR antagonists, M2 is a more potent ligand than the parent molecule and M1 has a slightly weaker binding affinity than vinclozolin in the same test (Wong et al. 1995). Although metabolism may therefore not be an essential prerequisite for the ED activity of vinclozolin, the metabolites contribute and depending on the predominant metabolite formed, there may even be an enhancement of the activity, in comparison to that of the parent compound.

The reports above show that a number of metabolites of substances are more potent than or at least as potent as EDs in comparison to the parent compound, and there are many more examples in the scientific literature, particularly for persistent organic pollutants including bacteriocides and pesticides. Also metabolism can convert agonists into antagonists. This suggests that inclusion of metabolism is needed in ED testing to increase the sensitivity of the test and to augment the reliability to predict the effect of a chemical. It could be argued that the tests which are presently available, would detect the parent compounds because of their relatively high sensitivity. However, because there is no information available on the metabolites of the majority of chemicals, it is not known how many are currently not being detected as EDs because the parent compound is not sufficiently potent. In the long term it might also become an advantage for accurate hazard and risk assessment if one could predict

the potency of the actually formed ED metabolites rather than the less potent parent compound. Furthermore, it may be of added value in terms of risk assessment to know that active metabolites could be formed, because these may prolong the action of a xenobiotic if the parent compound is not persistent and has a short half-life.

2.4 Potential problems with using exogenous metabolism in studies of endocrine disruptors

The paucity of published studies involving the use of exogenous metabolism in *in vitro* assays for EDs is surprising. It is recognised that there could be difficulties when adding exogenous metabolising fractions, e.g. due to non-specific hormone binding to protein in the enzyme fractions or to cytotoxicity of these fractions to target cells. Several EDs are known to bind strongly to serum proteins, and when this phenomenon occurs *in vivo*, it will affect internal target tissue concentrations of the chemical involved (Safe *et al.*, 1997). Such binding can occur with components of culture media or metabolising systems like S9, thereby resulting in both variable *in vitro* data, and differences in responses obtained in *in vivo* and *in vitro* assays. This phenomenon could be alleviated by removing proteins that are not necessary for testing from the medium. It is clear that protein binding of potential EDs will need to be carefully studied in the first place because it might be an important determinant of its *in vivo* activity, but also to better interpret results from *in vitro* studies. A study on 64 chemicals performed in a reporter gene assay making use of S9 indicated that there are two potential problems associated to the use of S9. Trans-stilbene was used as a reference agonist because it needs to be hydroxylated to trans-4-hydroxystilbene and trans-4,4'-dihydroxystilbene in order to be active. With the amounts of S9 required, estradiol at normally active concentrations was inactivated, while higher concentrations of estradiol were again more active with S9, than without. This is explained by an inversion of the concentration response curve that has a maximum at about 100 pM. The second problem that was encountered was the low reproducibility (Personal communication: Masahiro Takeyoshi, Gene analysis section, Hita laboratory, Chemicals Evaluation and Research Institute, Japan).

The logistical problems described above in using exogenous metabolism and developing new cell strains capable of acting as indicators of ED activity and of metabolising such chemicals in a relevant way have prompted a relatively recently published report (ICCVAM, 2003) on the use of *in vitro* binding and transcriptional activation (TA) assays for EDs, to conclude that '*the inclusion of a metabolic activation system in in vitro ER and AR binding and TA assays is not recommended at this time, as the type of metabolic activation system developed will depend on which in vitro assays are selected.*'

There is one other potential complication relating to what is known about the structural requirements for estrogenicity, in particular binding to the ER. As stated earlier, many estrogens contain one or more phenolic groups on a relatively small lipophilic molecule. C-hydroxylation on ring structures is precisely what occurs as a result of initial CYP monooxygenase activity on xenobiotics and this may result in many substances that will be detected as estrogens *in vitro*. *In vivo*, a number of these will be further metabolised and no longer be active. As a consequence, the use of metabolising systems that express predominantly phase I pathways, as opposed to phase II conjugation reactions, might be very prone to generating false positive data. This potential problem would need to be addressed.

2.5. Fetal metabolism and infant metabolism

It is important to realize that phase I oxidative enzymes may be expressed to a different extent during fetal life and also phase II enzymes are expressed differently in fetal life and during later stages (Hines and McCarver 2002; McCarver and Hines 2002)

Thus not only may the metabolism of xenobiotics be different in the developing fetus, but also the synthesis and degradation of steroids may be differ. As the fetus may be particularly sensitive to the effect of EDs (see for example McLachlan 2001 and the European Union's Environment and Health Strategy: SCALE, 2003 and Action Plan, 2004), future work to investigate the possible interactions of EDs with the CYP family in the fetus may be needed. There is experimental evidence for the presence of CYP1A1, CYP2C8, CYP2D6, CYP2E1,

CYP3A4, CYP3A5, CYP 4A1 and particularly CYP1B1 and CYP3A7 in the fetal liver (de Wildt et al. 1999; Hakkola et al. 1998).

Many CYPs are absent or barely detectable in the foetal liver, and develop postnatally. Three major stages in the expression of P450 enzymes can be discerned: CYP3A7 and 4A1 are the predominant CYPs expressed in the foetal liver and, a second group of early neonatal enzymes include CYP2D6 and 2E1 which surge within hours after birth although these proteins have not been detected in fetal samples. CYP3A4 and CYP2Cs rise during the first weeks after parturition, while CYP1A2 is the last isoform to be expressed in the human neonatal liver. Among phase II enzymes, epoxide hydrolase and glutathione S-transferase π are very active in the fetal liver, whereas glutathione S-transferases and UDP glucuronosyl transferases develop within 3 months after birth (Cresteil 1998).

2.6. Metabolism during digestion

The diet represents a major route of exposure to possible EDs and the chemical transformations which can occur during digestion further complicate considerations of metabolism. The myriad of processes involved in digestion have proven difficult to model, but two particular studies have emphasized the importance of examining these sorts of transformations in in vitro assays. First, nitrosylation reactions can occur in the saliva and acidic pH of the stomach through reaction of amines, phenols and mercaptans with sodium nitrite, a common food preservative and product of gut microbe-mediated reduction of nitrate (Assembly of Life Sciences., 1981, Chapters 4 and 6; Spiegelhalder et al. 1976; Tannenbaum et al. 1975; Challis 1973). The phenolic structure of several EDs makes them susceptible to nitrosylation, and although the reaction has attracted more attention as a mechanism of mutagenic activation, hormonal activity may also be modified. Nitrosylation of Bisphenol A, an estrogenic component of plastics and epoxy resins, causes both mutagenic activation and a decrease in estrogenicity as measured by green fluorescent protein reporter gene activity and ER α binding assays (Schrader et al. 2002; Masuda et al. 2005). A second effect of digestion on hormonal activity is evident in comparisons between the soy isoflavone daidzein and its metabolite equol. Equol is produced by intestinal

hydrolysis of the glycoside followed by bacterial biotransformation (Setchell et al. 1984, 2002) and has been shown through cell proliferation and receptor binding studies to be a much more potent ligand than the parent compound for ER α (Lehman et al. 2005; Setchell et al. 2005) and PXR (Jacobs et al. 2005), although it is metabolised far more rapidly than synthetic EDs, which are often more persistent.

3. THE METABOLISM OF ENDOGENOUS STEROIDS

3.1 Introduction

The steroid ring structure is a substrate for phase I metabolism by numerous CYP isozymes with multiple hydroxylations (Table 4) occurring at a number of preferential sites around the steroid molecule. Several of these reactions are concerned with the biosynthesis of different endogenous steroids from cholesterol via the formation of pregnenolone (See Fig. 2; Jacobs, 2004 and Fig. 3; Mensah-Nyagan et al. 1999). The initial biosynthetic reaction involves CYP-catalysed cleavage of the 6-C group from cholesterol, to form C-18, C-19 and C-21 steroid ring structures. Further reactions are catalysed by another isozyme (aromatase or CYP19), that converts the intermediate androgen structures in the biosynthetic pathway to estrogens via a process of aromatisation (thus the enzyme is known as aromatase). Other CYP isozymes are responsible for the activation or inactivation of the steroid substrate, as discussed below.

The various CYP isozymes responsible for hydroxylation are usually designated according to the substituent position that is hydroxylated. Also the enzymes exhibit stereochemical selectivity, with both hydroxylations being possible at the same or at different sites, depending on whether the resulting substituent is orientated above or below the essentially planar ring system. There are also some sex- and age specific differences in steroid metabolism (Jacobs and Lewis 2002), and whether or not animal models have been castrated or ovariectomised, can also affect such metabolism (Steimer, 2003).

A wide variety of CYP isozymes are able to hydroxylate steroids, although the most important groups of isozymes with respect to the quantity and variety of hydroxylations involved are the 3A and 2C isozyme groups (Table 4). CYP3A4 is known to be able to hydroxylate estradiol (Stresser and Kupfer, 1997). This enzyme also plays a prominent role in human the metabolism of xenobiotic chemicals and drugs. It is obvious that if hormones and xenobiotics are the substrate of the same enzyme there is a potential for complicated interactions. The enzyme may generate active metabolites from an inactive precursor or it may form inactive metabolites from an ED. The mere fact that xenobiotics, while being metabolised, are competing with endogenous substrate may also result in reduced biotransformation of the latter, which may induce ED effects. The degree of competitiveness or irreversibility of the interaction between a xenobiotic and the enzyme may be anticipated to be an important determinant of the effect. The interaction of xenobiotics with a number of key enzymes may be examined *in vitro* in a first attempt to determine its metabolic pattern and for this purpose genetically engineered cell lines which express CYPs can be used.

Estradiol and testosterone are the two most widely studied endogenous steroids with regard to CYP metabolism. The former has been studied extensively due to its use as one of the active ingredients of the female contraceptive pill and its potential association with the aetiology of breast cancer (Lord *et al.*, 2002). Estradiol is converted into numerous metabolites, the best known of which are estrone and oestriol. Estrone is further hydroxylated at the 2-, 4- or 16- positions by different CYPs (Table 4). Following estrone hydroxylation, further hydroxylations occur to form the respective poly-hydroxy derivatives that are then substrates for methylation or conjugation with glucuronic acid or sulfate. In a study involving human recombinant CYPs, it was shown that CYPs 1A2 and 3A4 exhibited the highest activities toward estrone (Shou *et al.*, 1997), while CYPs 3A5, 2C9 and 2C9R144C exhibited only moderate activities. The 2-hydroxylated product was formed exclusively by CYP1A2 and an unknown hydroxylation product was formed by CYP3A4.

Testosterone is hydroxylated by a wide variety of CYP isozymes at a variety of positions (Table 4). However, the major form of metabolism is 6 β - hydroxylation catalysed by

CYP3A isoforms (Lewis, 1996). The CYP2B isoforms generally exhibit regio-selectivity at the 16 and 17 positions of testosterone, with 2B1 exhibiting hydroxylase activity at the 16- α , 17- α and 16 β - positions. Testosterone is also hydroxylated at the 4 position by CYP2D6 (Dehal and Kupfer, 1997).

Interestingly, progesterone is not only subjected to 17-hydroxylation, but also has been shown to be metabolised to its 21-hydroxylated metabolite predominantly by CYP2C5 (Kronbach *et al.*, 1989). This enzyme exists as two isoforms in rabbits (Pendurthi *et al.* 1990) and similarity to CYP2C9 is suggested but remains to be confirmed (Afzelius *et al.* 2001, 2004).

Thus, the CYP enzymes exhibit catalytic activity toward a number of endogenous steroids, and this activity can be influenced by a number of factors, especially age, sex and disease, which are likely to be important in maintaining homeostasis in hormone levels at different times of development. Other important modulating influences on hormone metabolism include lifestyle, smoking, alcohol, diet and drug use and abuse, and individual differences due to genetic polymorphisms (Hatagima, 2002). The genetic polymorphisms that are most relevant to the enzymes that play a role in the metabolism of xenobiotics have recently been reviewed (Wormhoudt *et al.* 1999; Pelkonen *et al.* 1999).

Some chemicals that have estrogenic activity despite having low affinity for the ERs, such as hydroxylated PCBs and polyhalogenated aromatic hydrocarbons (PAHs), may exert this effect by inhibition of estrogen sulphotransferase, as discussed in section 2) thereby maintaining the available estrogen at higher levels (Kester *et al.* 2000, 2002; Shevtsov *et al.* 2003) as well as via the ER. Whereas Kester *et al.* used enzymatic assays, as with Warings group (Harris and Waring 2005) to investigate this possibility, Shevtsov *et al.* made a first attempt to study structural aspects using the crystal structure of the enzyme.

Other Detailed Review Papers are dealing in much more detail with the enzymes involved in the synthesis of steroids and with aromatases. It is however important to realize that there is

a link between the metabolism of xenobiotics and steroids through the many enzymes that participate in both pathways. In particular when developing tests to detect antagonistic effects of chemicals this is crucial since the natural hormone will be most likely used as the agonist and the incorporation of metabolic capacity is likely to have effects on the concentration of the agonist that should be taken into account while devising the test.

3.2 Metabolic activation of endogenous steroids

It is well-established that endogenous steroids can be converted to active metabolites and to inactive metabolites in target tissues (see Steimer, 2003 for a review). In the former case, as with many potentially toxic substances, some steroids have to be converted *in situ* to an active form before being able to interact with their respective receptors. This metabolic activation step is either an absolute pre-requisite, or a way of achieving a range of complex effects which give rise to interactions with more than one type of receptor.

A well-known example is the conversion of testosterone to 5 α -dihydroxytestosterone, a step that is necessary for its action on prostate growth, whereas its aromatisation to 17 β -estradiol in the brain is required for its developmental, neuro-endocrine and behavioural effects. Metabolic activation has been shown to play an important role in the activities of several other steroid hormones, including the progestins and androgens. Enzymes involved in metabolic activation of steroids usually catalyse irreversible conversion steps, and are often rate-limiting for steroid action (i.e. the effects depend more on the efficiency of metabolism than on the number of receptor molecules (binding sites) available).

Estradiol is converted by CYP enzymes to a number of biologically active metabolites, with some of them being estrogen antagonists. For example, while the 2-hydroxy metabolite of estrone is inactive, the 16 α -hydroxy metabolite is antagonistic, as are the 4-hydroxy metabolites, although they are produced in lower amounts.

There is much information implicating the formation of estrogenic metabolites of estradiol (such as 16 α -hydroxyestrone) as being carcinogenic and associated with the development of

breast cancer in humans (Lord *et al.*, 2002). The relative amounts of 2-hydroxyestrone and 16 α -hydroxyestrone can be measured in human urine by using an ELISA method, and the ratio of the latter to the former metabolite is elevated in patients with breast cancer, although the existence of this link is controversial (Safe, 2000). CYP1A1, which is inducible by active ingredients of cruciferous vegetables at high doses, is responsible for generating the 2-hydroxy metabolite. Cruciferous vegetables are known to reduce the risk of breast cancer. The compounds involved include indole-3-carbinol, a weak ligand in the AhR and one which may not ultimately induce CYP1A1 dependent enzymes. Observations reported in the literature suggest that the antiestrogenic effects of the active compounds in cruciferous vegetables may not necessarily involve altered CYP dependent estrogen metabolism (reviewed in Kim & Milner 2005). In addition to receptor cross-talk (Ohtake *et al.*, 2003, Jacobs 2005), other metabolic routes may include induction of glutathione-S-transferases, inhibition of other CYPs as well as weak estrogen antagonist activity (Michnovicz *et al.* 1997; Hecht 1999; Lampe *et al.* 2000; Meng *et al.* 2000; Rose *et al.* 2000; Chen *et al.* 2001; Nakajima *et al.* 2001; Sanderson *et al.* 2001; Ambrosone *et al.* 2003; Leibelt *et al.* 2003).

Testosterone is also metabolised to androstenedione by 17 β -hydroxysteroid dehydrogenase and by 3 β -hydroxysteroid dehydrogenase. A question that may need to be addressed is whether certain enzymes are specifically expressed in tissues like uterus, brain, testes, ovary and breast at a certain time in life and may be affected by some EDs with resulting changes in steroid levels and hence changes in hormonal balance at the local level, or on the other hand whether some of these chemicals might be metabolised by local CYPs with formation of locally active EDs. The breast is one of the tissues where local expression of CYP enzymes is described (Hellmold *et al.* 1995, 1998a,b; Stromstedt *et al.* 1993; Warner *et al.* 1997, 1998), which may be relevant to endocrine disruption because of its sensitivity to estrogens. Gender considerations in P450 metabolism are clearly an important consideration (Lewis 2003).

3.3 Metabolic inactivation of endogenous steroids

The metabolic inactivation of endogenous steroids occurs mainly in the liver, but also in the kidneys, and is required to ensure steady-state levels of plasma hormones, as steroids are more or less continuously secreted into the blood stream. There are five principal inactivation pathways, depending on the structure of the steroid substrate: a) reduction of the double bond at C4 and reduction of an oxo(keto) group at C3 to a secondary alcohol group; b) reduction of an oxo group at C20 to a secondary alcohol group; c) oxidation of a 17 β -hydroxy group; d) further hydroxylations at various positions of the steroid nucleus; and e) conjugation to form sulfate or glucuronide derivatives.

Other extrahepatic tissues that may be important to consider are the skin and adipose tissue. The skin is particularly active in metabolising endogenous and exogenous steroids (the latter being applied topically as medicines for example) (Hotchkiss, 1992). The cutaneous metabolism of steroids involves the co-factor-dependent hydroxysteroid dehydrogenases (Penning, 1997) which interconvert keto- and hydroxy- groups, and the 5 α -reductases. The dehydrogenases catalyse the interconversion of testosterone and androstenedione, estradiol and estrone, as well as cortisone and hydrocortisone (cortisol). Also, the 7 α -hydroxylation of dehydroepiandrosterone, and the hydroxylation of testosterone at the 7 α -, 16 α - and 6 β -positions have been demonstrated, suggesting the activities of *CYP 3A* and *2C* isozymes. RT-PCR analysis of proliferating keratinocytes from the human epidermis from 5 male donors revealed constitutive expression of *CYP1A1*, *CYP1B1*, *CYP2E1*, *CYP2B6*, and *CYP3A5*. Expression of *CYP3A4* could be induced by dexamethasone (Baron et al. 2001). By using microsomes prepared from human keratinocytes, *CYP1A1*, *CYP2B6*, *CYP2E1* and *CYP3A* could be determined by immunoblotting. These enzymes were also detected by immunofluorescence of differentiated keratinocyte multilayers and the respective enzyme activities could be detected in microsomes from human keratinocytes (Baron et al. 2001). Yengi et al. (2003) investigated 13 P450 enzymes by quantitation of P450 mRNA. In RNA isolated from human skin biopsies, *CYP* mRNA of the following enzymes could be quantified by RT-PCR in human skin samples from 27 healthy volunteers: *CYP1B1*, *CYP2B6*, *CYP2D6*, and *CYP3A4*. Lower levels were found for *CYP2C18*, *CYP2C19*, and

CYP3A5, whereas CYP1A2, CYP2A6 and CYP2C8 were below limits of detection. Smith et al. (2003) investigated CYP2S1 expression in human skin biopsy samples. It could be demonstrated by RT-PCR of CYP2S1 mRNA, by immunohistochemistry and by metabolism of all trans-retinoic acid, that CYP2S1 is constitutively expressed in human skin. Cutaneous expression of CYP2S1 is inducible (for example by ultraviolet radiation or coal tar). It has been demonstrated, that CYP2S1 expression mainly occurs in epithelial cells (Saarikoski et al., 2005). Saeki et al. (2002) investigated mRNA from cultured Langerhans cells, keratinocytes, fibroblasts and melanocytes from 6 individuals. CYP1A1, CYP1B1 and CYP2E1 were found in all four cell types. CYP2A6, CYP2C, CYP2D6, CYP3A5, CYP3A7 and CYP4B1 mRNA was expressed in a cell –type – and or individual specific manner. CYP1A2, 2A7, 2B6 and 3A4 mRNA was not detectable. In a recent review by Du et al. (2004) it has been summarized, that at least 13 different CYP2 genes (CYP2A6, CYP2A7, CYP2B6, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1, CYP2J2, CYP2R1, CYP2S1, CYP2U1, and CYP2W1) are expressed in skin from at least some individuals and that the majority of these genes is expressed in the epidermis or in cultured keratinocytes.

3.4 *The consequences of metabolism for hormonal activity*

It is well known that the affinity of steroids for their respective receptors is crucially dependent on the presence of specific functional groups, as well as on 3-D structure. Minor changes in these properties, for example as a result of metabolism, can drastically modulate binding affinity. Thus, it follows that target tissue metabolism can play a critical role in the activity of steroids.

4. THE TESTING OF ENDOCRINE DISRUPTORS AND METABOLISM

4.1 *Introduction*

Some substances like methoxychlor are relatively well studied with regard to metabolism and the hormonal activity of its metabolites (see section 2.3). Obviously the substances with hormonal activity that are used in medicine are also usually well documented. However substances with ED effects that are present in the environment are often less well studied.

There are a few studies available with some other substances than those mentioned in section 2.3.

A human microsomal metabolising system was used by Vakharia & Gierthy (1999) to generate hydroxy metabolites of some PCBs. The estrogenicities of the resulting metabolites of some of these parent compounds were determined by their abilities to bind to the ER. In some cases there was evidence for metabolic activation. In a later study, it was shown that it is possible to incorporate a post-mitochondrial supernatant fraction (S9) into an assay involving a yeast hybrid system (Joyeux *et al.*, 1997). There was evidence for the activation of some of the chemicals (13 bisphenol A-related chemicals) by S9 (Hashimoto *et al.* 2001). Yoshihara *et al.* (2001) used a yeast hybrid assay and a mammalian cell system (the MCF-7 cell line) to demonstrate that rat-liver S9 was able to enhance the estrogenicities of bisphenol A, bisphenol B and also methoxychlor. On the other hand 4-*tert*-octylphenol, 4-nonylphenol and 17 β -estradiol were inactivated when metabolising capacity was added. These authors showed that the activating effects of S9 were due to CYP activity as they were abolished by SKF-525-A. They also found that neither cytosol nor microsomes alone were able to activate the chemicals. In a later study they indicated that also human, monkey and mouse S9 could form metabolites that are more active than the parent bisphenol and in particular 4-methyl-2,4-bis(p-hydroxyphenyl)pent-1-ene should be mentioned (Yoshihara *et al.* 2004). This work needs to be confirmed and extended, and the issue of protein binding examined directly. Also, different types of enzyme fraction need to be used with sub-cellular receptor binding assays, as well as the use of different mammalian indicator cell lines.

The same group (Kitamura *et al.*, 2003) have more recently extended their studies to a group of styrene oligomers by using the same biological systems in the presence of rat liver microsomes. They showed that *trans*-1,2-diphenylcyclobutane (TCB), 1,3-diphenylpropane and 2,4-diphenyl-1-butene were only activated when microsomes from phenobarbitone-induced rats were used, and when incubations were conducted in the presence of NADPH. The active metabolite of TCB was identified by HPLC as its 4-hydroxy derivative. Thus, these results differ from the former ones obtained by these authors in that microsomal

fractions were able to activate the chemicals. Unfortunately, they did not specify whether an inducer had been used in the earlier experiments. Meerman et al (2003) have also shown that rat liver microsomes can be used to activate several polycyclic aromatic hydrocarbons to metabolites that can bind to the ER in binding, cell proliferation and reporter gene assays. More recently this group have also shown that dibrominated biphenyls are bioactivated by CYPs to metabolites with estrogenic activity and inhibiting estrogen sulphotransferase (hEST) (van Lipzig et al.2005).

Using rat hepatocytes, bisphenol A glucuronide could be detected as a major metabolite (Elsby et al. 2001b). Glucuronidation by microsomes prepared from human livers was considerably less than that by immature female rat microsomes (Elsby et al. 2001b). Estrogenic activity of bisphenol A was determined in a yeast assay coupled with microsomal metabolism and estrogenicity was decreased less by the human microsomes than by the immature rat microsomes (Elsby et al. 2001b). This indicates that species differences need to be addressed and the physiological relevance of using rat or human systems should be established. In addition it was shown that in humans, bisphenol A may be rapidly excreted as glucuronidated metabolites (Völkel et al. 2002, 2005).

Although some tests to measure endocrine effects and in particular estrogenic potency do already include assays for metabolism, these have not been (pre) validated and thus can be considered as being preliminary for regulatory purposes. They suggest however that tests designed to investigate both metabolism and an endocrine target simultaneously, are both possible and important for human hazard assessment.

4.2 Possible ways to overcome problems of using exogenous metabolising systems

The presence of serum proteins that may not only bind steroids, but possibly also exogenous EDs, may hamper the use of metabolising cell free systems or cells with metabolising capacity in conjunction with indicator cells that contain the endocrine target. This issue should be addressed carefully during the standardization and validation procedure. It may after all be advisable to incorporate a mandatory *in vitro* test to measure plasma protein

binding of chemicals in future toxicological testing schemes. If protein binding does turn out to be problematical, then a potential solution would be to rely on the metabolism of the indicator cells. As discussed earlier, there are two ways of achieving this, either: a) to hope that the cells that are used in the assays have sufficient inherent and relevant enzyme activity; or b) to generate metabolically competent cell lines via the introduction of the genes for enzymes into the cells and by using culture conditions in which the enzymes are expressed. In both cases, intracellular metabolites can be generated *in situ* close to or at the sites of the relevant receptors.

4.2.1 The intrinsic metabolising capacity of cell lines used for testing endocrine disruptors

There is little known about the intrinsic metabolic competence of the usual mammalian cell lines that are used for detecting EDs acting via receptor binding, and such an investigation needs to be undertaken. In this context, it is of interest that discrepancies in the estrogenicity of methoxychlor (discussed above) in various *in vitro* assays have been attributed to differences in the metabolic competencies of the various indicator cells involved (Andersen *et al.* 1999). Thus it was assumed that some of the cells involved possessed some residual demethylating activity. Also, evidently MCF-7 cells are capable of generating the normal hydroxylated metabolites of estrone that are found *in vivo* (Bradlow *et al.*, 1995), although their ratios were not the same in relation to breast cancer incidence as those found allegedly *in vivo*.

The benzoate esters of both nonyl phenol and estradiol were both active in a rodent uterotrophic assay, but inactive in a yeast cell assay, probably due to low levels of ester hydrolysis *in vitro* (Odum *et al.*, 1997). These results could be explained if yeast cells possess selective esterase activity, and could account for the discrepancies between *in vitro* and *in vivo* data observed for the same chemicals.

It is of course feasible that false positive data could be generated *in vitro* due to a lack of detoxification. In this context, it is of interest that some phthalate metabolites, such as mono-butyl phthalate and mono-benzyl phthalate, which were estrogenic in a recombinant yeast

screen, were inactive *in vivo* (Harris *et al.*, 1997). By contrast, the inactivity of certain chemicals *in vitro* (e.g. some PCBs in the MCF-7 cell proliferation assay) might be due to an inability of the breast cancer cells in culture to convert the chemicals to reactive forms.

There is also some information concerning a human adrenocortical carcinoma cell line (H295R), which is being used to detect chemicals that affect steroidogenesis (hormone synthesis) (Johanssen *et al.*, 2002). This cell line expresses CYPs 11A, 17, 21 and 11B1.

4.2.2 The potential to use genetically-engineered cell lines

In addition to the many cell lines that have been genetically engineered to express CYP enzymes and various phase 2 enzymatic reactions, there are several genetically-engineered mammalian cell lines that have been developed to express human or mammalian nuclear receptors for a variety of steroid classes (e.g. estrogens, prostagens and androgens). These receptors are expressed in conjunction with their respective response elements linked to promoter regions for a reporter gene, together with the reporter gene itself. Unfortunately, none of these cell lines were also transfected for metabolising enzymes, and yet some of them (e.g. COS and Hep G2) have been used to study metabolism or endocrine disruption in separate studies. However, Mak *et al.*, (1999) have developed a novel *in vitro* screen for aromatase inhibitors that involves the use of a yeast strain that co-expresses human aromatase CYP19 and the mouse AR receptor as well as an androgen-responsive β -responsive reporter plasmid.

One of the problems that were identified at a recent ECVAM workshop is the limited number of CYPs that can be incorporated into one cell line. This would mean that at present it will remain difficult or even impossible to study the impact of a chemical upon a chain of metabolising enzymes where the reaction product of the one is the substrate for the other, as was demonstrated for methoxychlor (see above), in genetically engineered cell lines.

Similarly it would be difficult at present to study the impact of metabolism on the endocrine effect of a chemical in genetically engineered cells, if several enzymes could form the same or even different but equally active metabolites.

5. Proposals to develop a framework for the simultaneous testing of endocrine disruption and metabolism

5.1 Tiered approach including determination of the likelihood of systemic exposure

In order to replace the animal testing of cosmetics by *in vitro* tests, a tiered approach was proposed for these substances. In a first tier the likelihood of systemic exposure would be predicted from dermal absorption tests, (Q)SPR approaches, kinetic modeling and cell based assays (ECVAM 2003). A tier 2 battery was proposed consisting of (Q)SPR approaches, plasma protein binding, kinetic modeling, blood/tissue partitioning tests and metabolism (biotransformation) assessments (ECVAM 2003). Substances that will likely be systemically available will be used in the third tier, which involves toxicity testing of the substances with determination of their potency (ECVAM 2003). One option could be to prioritize potential EDs and limit further testing to those compounds that are likely to be absorbed using the first tier of the approach for cosmetics. For cosmetics the main route of exposure may be through skin absorption because of the way these chemicals are used. EDs may enter via the oral route (e.g. food, drinking water), via inhalation (e.g. the spraying of certain pesticides) or through the skin (e.g. cosmetics). In order to fully rely on these approaches, all routes of exposure thus need to be extensively covered by rigorously validated methods. Substances that could be taken up by neonates however should be tested generally because the intestinal barrier in neonates may be permeable to compounds that would not pass through the intestinal barrier at a later age. In particular for persistent substances like certain pesticides, it may be important to consider the site in the organism where the parent compound is stored, where the metabolites are formed and where those metabolites might be stored in turn. A second tier for ED screening could then take into account the potency of the chemicals at hormonal targets, together with *in vitro* data on metabolism obtained separately, in order to further prioritize potential EDs for *in vitro* testing. As the development and validation of methods to specifically detect endocrine disruption is ongoing at OECD we refer to this effort for further details. In later stages when tests will be available that can take into account metabolism and endocrine targets simultaneously, these should be preferably used.

Evidently, any information obtained from *in vivo* testing could also be used in a final framework for the combined testing of ED activity of chemicals and of their metabolism. Such information may be obtained from assays that are specifically aiming at endocrine endpoints like the uterotrophic assay or the Hershberger assay that are being validated, but it may also be obtained from other validated toxicity assays.

5.2 *In vitro* methods to study metabolism

5.2.1 Screening tests for metabolism

In order to identify the most important metabolic pathways, human liver fractions or genetically-engineered cell lines could be incubated with the chemical, followed by quantitative analysis with techniques such as liquid chromatography (LC) and mass spectrometry (MS) (Doehmer et al., 1999; Kassahun et al., 2001). The separation and identification of metabolites by HPLC or GC would be more time consuming than the application of modern approaches like LC/MS/MS.

A priority put forward at a recent ECVAM workshop is the development of a general test to determine *in vitro* metabolism. This approach would allow the detection of compounds that are unlikely to be metabolised and for which metabolism may reasonably be excluded as a mechanism contributing to toxicity. Nevertheless it can be anticipated that the largest part of chemicals will be metabolised. If metabolites can be identified and isolated, or synthesized in sufficient quantities, they could be tested in assays that aim to detect ED activity (Freyberger and Scholz 2004). At present this approach may not be possible for large scale screening.

To screen for metabolic stability, i.e. the property of a compound not to be metabolised, it is possible to use microsomes, human hepatocytes or genetically-engineered cell lines expressing human biotransformation enzymes. These approaches involve the incubation of the chemical with the cells or microsomes, followed by enzyme kinetic studies based on chromatographic procedures, such as high performance liquid chromatography (HPLC) and LC-MS (Linget & Vignaud, 1999). The exclusive use of microsomal preparations may give

misleading results, because phase II enzymes, which are predominantly cytosolic enzymes playing a crucial role in the metabolic biotransformation of chemicals, may be missing. In some cases, this problem can be solved by the exogenous addition of cofactors such as UDP-glucuronic acid (UDPGA) for glucuronidation, and 3'-phosphoadenosine-5'-phosphosulphate (PAPS) for sulphation (Swales & Utesch, 1998). Whether the addition of other factors such as glutathione and acetyl CoA are required should also be considered.

For the assessment of inhibitory interactions with CYP enzymes, the most popular approaches involve the use of genetically-engineered cell lines containing only one specific human CYP isoform (Coecke et al., 2000) in culture or microsomes thereof (Miller V.P., et al. 2000). The effect on the biotransformation of the substrate is dependent on the concentrations of both the inhibitor and the substrate, determined by the inhibition constant, K_i , of the inhibitor and the Michaelis-Menten constant, K_m , of the substrate. The test compounds are incubated with the cultures or the microsomes in the presence of CYP substrates. Quantification can then be performed by appropriate fluorescence analysis which would probably be the most time saving method especially when performed in microplates, HPLC or LC-MS. In situations where more than one enzyme isoform is present, the use of complex *in vitro* models, such as human hepatocyte cultures (Li et al., 1999) is advised, so that inhibition and metabolism can be evaluated at the same time.

The use of S9 in combination with an assay for endocrine disruption testing may have a number of shortcomings. In cases where an animal source of S9 is used, there might be important species differences with regard to prediction of human toxicity. When human sources of S9 are available, the amounts are low or limited. Even in cases where a human source of S9 would be available in sufficient quantities for screening, the matter of a reproducible degree of induction and the potential large experimental variability will probably remain an issue to be resolved. Some preliminary findings support the idea that human S9 can be used in practice (Yoshihara et al. 2001, 2004). It may be worthwhile to search for sources of human S9 and investigate their potential usefulness for incorporation into *in vitro* screening tests. Despite possible difficulties and shortcomings, incorporation of

S9 in ED testing is a likely alternative for inclusion of metabolic capacity for large scale testing in the near future. There is some literature that demonstrates the possible usefulness of a combination of S9 and ED targets (see section 1.4.3, 2.3 and 4.1), and it is a validated means of introducing metabolism in the context of mutagenicity testing. It may also be a way of integrating metabolic capacity into tests with other endpoints than those which are envisaged for ED testing now, according to future availability. It would be necessary to establish the importance of the incubation time with S9, since this will not only determine the amount of metabolite formed, but it may also have an impact on cell toxicity.

5.2.2 Induction of biotransformation enzymes

Xenobiotics taken up by the human body may induce de novo synthesis of enzyme molecules (including phase I and phase II biotransformation enzymes) as a result of increased transcription of the respective gene. An increase in enzyme activity may also be observed as a result of enzyme stabilisation (Coecke et al., 1999).

Various *in vitro* metabolically-competent models have been proposed for the detection of the induction of CYPs, including precision-cut liver slices, short-term and long-term hepatocyte cultures, liver-derived cell lines expressing or re-expressing biotransformation enzymes, and highly differentiated human cell lines (Coecke et al., 1999, Gomez-Lechon et al., 2001). For detecting enzyme induction, these *in vitro* methods involve the use of endpoints such as: a) the activity of the biotransformation enzymes (and, if appropriate, their individual isoenzymes); b) protein levels, by using techniques such as immunoblotting or HPLC; and c) mRNA levels, by using Northern blotting, e.g. via the nuclease protection assay or the reverse transcriptase polymerase chain reaction (Coecke et al., 1999).

Although there are technical difficulties associated with the cryopreservation of hepatocytes, a number of cryopreserved hepatocyte cultures have been used for enzyme induction studies (Hengstler et al., 2000). To date, no prevalidation study has been carried out on methods for identifying the capacity of compounds to induce biotransformation enzymes. However, ECVAM initiated a prevalidation study (Coecke et al., 1999) on the use of human hepatocyte sandwich cultures (LeCluyse, 2001).

Since many hormones - in particular those that are at present considered most prone to endocrine disruption - act on NRs that regulate gene expression, EDs can and may also interact at this level, inducing and inhibiting enzyme mediated pathways, beyond the ER and AR. While awaiting tests that can reliably determine the potential of enzyme induction *in vitro*, it might be worthwhile to validate existing ligand binding and transfection assays for AhR (Seidel et al. 2000; Ziccardi et al. 2000; Nagy et al. 2002; Rogers and Denison 2002), CAR and PXR. For instance phthalic acid and nonylphenol, but not bisphenol A increased transcription related to PXR activation and increased CYP3A1 mRNA in rat liver (Masuyama et al. 2000). Two non-hydroxylated parent PCBs (PCB 118 and 153), trans-nonchlor and triclosan have also been shown to activate the human PXR *in vitro* (Jacobs et al. 2005). This would not only identify potential enzyme inducers that require further investigation, but also potential EDs that interact with these receptors and that otherwise would not have been detected.

5.2.3 Models for evaluating polymorphic effects on metabolism

Amino acid substitution or deletion due to genetic variation can result in reductions, or even loss of the activities of phase I or phase II biotransformation enzymes. Certain individuals exhibit a severely compromised ability to metabolise chemicals that are specific substrates of these polymorphic enzymes, which can lead to serious toxic side-effects (Wolf & Smith, 1999).

The acetyltransferases, NAT1 and NAT2, represent the best-understood polymorphic enzymes. Other phase II enzymes, such as SULTs, UGT, and GST, are known to exhibit a variety of polymorphic variants, and understanding of their functional genetic diversity and relevance to ED mechanisms is improving (Burchell et al., 1998, 2000; Eaton & Bammler, 1999; MacGregor et al., 2001; Glatt, 2000). For example, as discussed earlier, SULTs are an important route for steroid modulation that can be modulated at physiological concentrations. (Waring & Harris, 2005).

Although genetic polymorphisms of metabolising enzymes are considered as being potentially important for endocrine disruption because it might make a group of people more or less susceptible for the enzymes' action, it may not constitute the highest priority to establish the possible hazards that chemicals have to induce hormonal effects. Genetic polymorphism is probably more important for epidemiological monitoring.

5.3 Approach to combine the *in vitro* study of endocrine disruption and metabolism

If hormonal targets were to be used exclusively for screening, there is a high likelihood that one would miss a number of EDs that require enzymatic activation. It would also be possible that some false positives would be detected since compounds that would be very rapidly metabolised and thus be inactive *in vivo* might be active on an *in vitro* target when studied in the absence of metabolism. Although this might be acceptable in a testing scheme to detect hazards, it is unacceptable for the performance of risk assessments. Furthermore, false positives might incur large and unnecessary economic costs. The most efficient way to effectively lower risks of exposure to unknown EDs might therefore be to develop a framework that combines *in vitro* tests on both metabolism and hormonal targets.

A major point with regard to validation of assays will be to make sure that the metabolic competence that will be applied to an *in vitro* system simulates the mix of diverse enzymes that is of importance *in vivo*, and that the delicate balance between activation and deactivation of compounds that may exist *in vivo*, is preserved.

In the short term, the quickest and easiest way to implement such test may be to incorporate S9 into acceptable indicator tests for endocrine disruption in a fashion similar to the testing for mutagenicity. Possible problems like protein binding, the potential influence of the incubation time with S9, and cytotoxicity of S9, and substances used to induce enzymes in the S9, should be investigated and solved. It will be imperative to be able to reproducibly activate the enzymatic system. The importance of the species originating the S9 should be addressed. If the risk assessment for humans is the ultimate goal, then a human based system

is preferred. If prediction of risk for wildlife is the aim, then another species may be a better source for *in vitro* studies. Due to the potential problem of standardization of S9 from human origin and because further testing may involve tests on laboratory animals, another mammalian S9 such as that isolated from rats may also be considered. The intention of proposing the use of S9 is to reduce at this stage the potential of false negative results, but validation should take into account that not too many false positives would be detected because that would lead to unnecessary further testing. On the other hand, care should be taken that the metabolic capacity *in vitro* would not be too high in comparison to the metabolic activity *in vivo*, since otherwise active compounds may not be considered as an ED only because of an unrealistically high degradation of the xenobiotic in the *in vitro* test. While the use of S9 is a useful possibility that is available at present, efforts should be made to explore more refined methods that may mimic the *in vivo* situation more precisely.

Although the ideal solution for the simultaneous testing of ED potential and metabolism may not be reached in the near future, this should not prevent the use of the best possible combination of tests that are already available. If agreement on a framework for testing can be obtained, this might also be helpful to identify those tests that may be of utmost need and thus should be developed with the highest priority. It is suggested that primary *in vitro* tests (further abbreviated as P) for endocrine disruption at different targets, as described in chapter 1.4, should be developed and validated. These may not remain limited to the ER α and the AR ligand binding assays or to functional assays but could also include the other NRs of the same family like the ER β , progesterone, glucocorticoid and mineralocorticoid receptors and the thyroid receptors. While devising such tests, preference should be given to those tests that may be combined most easily with a simultaneous assessment of metabolism of the substance under study. Whether or not these receptors should also be incorporated in the battery of necessary tests would deserve further investigation. Chemicals that can interact with FSH and LH function, can interact at different levels with sex steroids and reproductive function. Therefore these two hormones should also be integrated into the framework. It seems imperative that functional tests will be developed to discern between agonists and antagonists. The combination of tests for antagonism with metabolic capacity will need

particular attention, since the concentration of the agonist used may be affected by the presence of metabolising enzymes. *In vitro* tests to detect metabolism of chemicals (further abbreviated MC) could be developed along the lines discussed in chapter 5.2, but evidently any information on metabolism that is available from other assays could be used at present to decide about the potential ED effects of certain chemicals. Thirdly *in vitro* tests to detect the interference of chemicals with steroid hormone synthesis and metabolism (further abbreviated as MH) should be developed but this will be the subject of two separate Detailed Review Papers. While the proposed tests would be directed to the testing of the effect of chemicals on ED targets, metabolism of the chemical or effect of the chemical on steroid metabolism separately, the same tests could in the end be used to combine the testing of the influence of chemicals on ED targets in conjunction with the testing of its effect on steroid metabolism (further abbreviated as PMH) or in combination with the testing of its own metabolism (further abbreviated as PMC). In particular the combination of S9 and a primary test may offer a valuable alternative in the short term. Furthermore validated *in vivo* tests for general toxicity (further abbreviated as G and including OECD test 407 for 28 day oral toxicity enhanced for ED effects, OECD 415 one generation reprotoxicity and OECD 416 two generation reprotoxicity) or *in vivo* tests with rather specific ED endpoints (including the uterotrophic and Hershberger assays) could offer information about ED effects.

A framework (Table 6) is proposed that would allow some prediction of the ED potency of chemicals while awaiting the availability of validated tests. For the time being, the development and validation of tests detecting a hormonal effect or metabolism may be simpler than the combination of both. At this moment they may offer a reasonable alternative for testing while awaiting combination tests. When a chemical would be inactive in all relevant P, MC and MH tests, one can anticipate that it will be probably devoid of ED activity but great care should be taken to avoid the occurrence of false negatives. If a more affirmative denial of ED effects is required at this stage, using *in vivo* tests may still be required.

When a chemical would have an effect in a test that was developed for the measurement of steroid metabolism, it can be considered a potential ED. The potency of the substance required to reach this decision would still need to be established. Further testing *in vivo* may be required to reach a more definite conclusion or to perform a risk assessment. To exclude potential species differences, an animal species known to be sensitive to the endocrine mechanism involved to the same extent as humans and for which the *in vitro* activity was confirmed, should be used. If this condition is fulfilled, a chemical that would be inactive *in vivo*, could be considered devoid of ED activity despite its effect on hormone metabolism *in vitro*.

When a chemical would be active in one of the tests developed to study its metabolism, it should be evaluated in the primary tests for ED after adding metabolising capacity, preferably utilizing the same metabolising entity where it was found active if this is compatible with the endocrine target to be tested. If the chemical would be inactive or very weak it is likely not an ED. If the chemical would be active as an ED in the presence of metabolising capacity, it would need to be considered an ED and *in vivo* testing may be required to reach a more definite conclusion for risk assessment. To exclude potential species differences, an animal species known to be sensitive to the endocrine mechanism involved to the same extent as humans and for which the *in vitro* activity was confirmed, should be used. If this condition is fulfilled, a chemical that would be inactive in an *in vivo* test that is sensitive to effects caused through the target that was affected *in vitro*, could be considered devoid of ED activity despite its activity in a primary test for ED with additional metabolic competence. Obviously, a scientific validation procedure should minimise the potential to detect such false positives *in vitro*. The potency limits determined by *in vitro* tests that would support the decisions to be made still need to be established.

When a chemical would be active in an *in vitro* test for an endocrine target it should be tested further in the presence of metabolic capacity. If it would not be active in same test when combined with metabolising capacity, it may be considered as probably not an ED because of metabolism. To be able to draw that conclusion, it would be imperative to

ascertain that the *in vitro* system has a metabolising capacity that is representative for the *in vivo* situation. If a chemical would remain active in the presence of metabolising capacity, it should be considered a potential ED. Subsequent testing in an appropriate *in vivo* test may facilitate a more validated conclusion of relevance to a risk evaluation. To exclude potential species differences, an animal species known to be sensitive to the endocrine mechanism involved to the same extent as humans and for which the *in vitro* activity was confirmed, should be used. If this condition is fulfilled, a chemical that would be inactive *in vivo* could be considered devoid of ED activity, despite its *in vitro* activity. Obviously a validation should aim at reducing the number of such false positives *in vitro*.

In the ideal situation, *in vitro* testing should precede any *in vivo* testing. However where available, information from *in vivo* tests is of value. If a chemical would be active in one of the general toxicity tests and the available information would suggest an endocrine mechanism of action, this can be further investigated *in vitro*. A positive test result *in vitro* would indicate that the chemical is an ED.

It does not appear likely at present that a chemical will be tested in an *in vitro* assay that would combine a primary endocrine endpoint and metabolism of hormones. However, in the event that this might be possible, any active compound should be treated in a similar way as one that was found active in separate *in vitro* assays for a primary endocrine endpoint and metabolism of the chemical. In this respect it should be noted that the PMH tests are considered less urgent to develop while the MH and PMC tests could be considered priorities as they can facilitate most of the conclusions necessary within the context of the proposed framework.

The ultimate goal should be to develop tests that would allow implementation of a flow chart for ED testing that would start in a first tier with *in vitro* assessment of systemic availability. If there is sufficient evidence to believe that a substance will not be systemically available and if it cannot be swallowed by mouth by neonates, the substance can be considered as being devoid of ED activity. If there is doubt or relatively large certainty that it

can be systemically available or if it can be swallowed because of its use by neonates, it should be further tested. A second tier should consist of tests for interference by chemicals with metabolism or synthesis of hormones and tests for endocrine activity in the presence of metabolising capacity. At present most attention is given to estrogenic, androgenic and thyroid activity but other hormonal and ED pathways may be envisaged as well. If the substance would not be active below an agreed and defined concentration in both kinds of tests, the substance can be considered to be devoid of potential ED activity. If there is activity in the second tier *in vitro* tests, the substance should be tested further *in vivo*. Possible third tier tests could include an enhanced OECD 407, OECD 415, OECD 416, uterotrophic assay or Hershberger assay or any other test that might become available and be considered relevant in the future.

6. CONCLUSIONS AND RECOMMENDATIONS

Clearly, it is crucially important that *in vitro* methods for testing EDs should take account of the need to provide a metabolising system, either in the form of subcellular enzyme fractions, or by using cells that have been rendered metabolically competent. This is based on the fact that there is sufficient information showing that endogenous steroids and EDs are extensively metabolised by phase I and II enzymes in both the liver and hormonally-active tissues, and that such metabolism can lead to the activation or inactivation of steroids and, with regard to ED's to detoxification but also to the formation of active metabolites. Despite the fact that there are several technical problems with using sub-cellular metabolising systems in assays for testing EDs, there is sufficient evidence in the literature to show that these limitations can be overcome and that the presence of such enzymes modulates the chemical toxicity of several different types of EDs.

The absence of metabolism in *in vitro* assays for EDs could give rise to false positive data (due to lack of detoxification) or negative data (lack of activation) arising from the use of the tests. This situation regarding the lack of metabolism being incorporated into *in vitro* assays for EDs is equivalent to embarking on developing and validating the Salmonella mutagenicity assay (Ames Test) without investigating the need to add liver S9 or

microsomal fractions. Clearly, if this course of action had been followed, the test would never have been accepted for mutagenicity, and as a screen for carcinogenicity. It is, therefore a serious drawback that currently the use of *in vitro* assays for EDs is not receiving the attention that it deserves, and this will only be rectified when the issue of incorporating aspects of metabolism into the assays has been achieved. The contribution of incorporation of metabolism into ED testing would be to avoid both false positives and false negatives, to be able to make risk assessments with *in vitro* data on the potency of the chemicals that matter more than the parent compound and to better estimate the possible duration of action. On the other hand, the development of a system for ED testing incorporating metabolism would require careful consideration so as not to generate an unreasonable and unjustified number of false positives and false negatives.

Thus, the perceived difficulties of incorporating metabolism into *in vitro* tests for EDs should not deter more research into how this can be achieved. The development and validation of these *in vitro* assays in conjunction with appropriate metabolising systems should be a matter of urgency.

It is recommended that approaches should be developed that can predict systemic exposure. Particular attention should be given to the potential oral exposure of neonates. If no systemic exposure is expected, chemicals could be regarded as being devoid of ED effects. Obviously not only tests but also criteria that would allow interpretation of the results should be provided. The possibility to use (Q)SARs for the analysis of potential ED effects through well specified mechanisms and/or for the analysis of potential interference with the metabolism of endogenous hormones and exogenous compounds, as well as the for the prediction of metabolism of chemicals by phase I and II enzymes, should continue to be explored and developed. At present there are not yet validated *in vitro* tests incorporating metabolic competence to measure the effect of chemicals on endocrine targets *in vitro*. Even separate testing for endocrine disruption or the potential of chemicals to be metabolised or to interfere with metabolism is not yet sufficiently validated. For the time being available data from *in vitro* tests for endocrine action and from metabolism tests could be used to further

prioritization of potential EDs for further testing. It would be advisable that criteria should be put forward to take this decision. In the future, tests should be made available that combine both testing for metabolism and endocrine effects and studies should be initiated that will gain more knowledge about the incorporation of metabolic capacity in existing functional tests or the incorporation of endocrine molecular targets into existing cell lines with metabolic capacity.

In the short term inclusion of S9 into *in vitro* ED tests may be the only reasonable alternative that could be made available. In the long term, it is recommended that reports in preparation concerned with the generation of genetically-engineered mammalian cell lines containing steroid hormone NRs, their response elements and reporter genes, together with genes expressing specific metabolising enzymes are assessed for feasibility for high throughput screening and subsequent submission for (pre) validation purposes. Such multiple expressing cell lines could then be utilised to investigate the metabolic activation and detoxification of potential EDs *in vitro*. Whether a substance is an ED or not can currently only be definitely established by *in vivo* experiments, however *in silico* and *in vitro* screening with the incorporation of metabolism would result in a more adequate prioritisation for further testing. It would indeed become possible to test the most reasonable dose or dose-range of the compounds that would be most likely to produce relevant effects. If a substance is suspected of being a potential ED on the basis of *in vitro* tests using enzymes and molecular targets of human origin, and the substance is going to be finally tested *in vivo*, it is advised to first confirm activity in tests *in vitro* with materials obtained from the animal species and strain that will be used for the *in vivo* test.

The assessment of intrinsic metabolising capacity of the cells used in ED testing is very important. On the one hand it could be used to a certain extent as a source of metabolic capacity; on the other hand it would constitute an inherent part of the test system that needs to be well described in order to correctly interpret results obtained. It may be worthwhile to identify substances that increase in endocrine disruption capabilities compared to those that become inactivated by metabolism. If one could construct a list of parent compounds and their metabolites with known effect on an endocrine disrupting target, one could pre-screen

in silico and test all of these substances in any new *in vitro* test that is being validated for endocrine disruption. Thus it might be possible in a short time frame to have a first indication of the metabolic capacity of the cells used in the test and thus the possible relevance of that residual metabolic capacity to develop a combined test.

It is also recommended that there should be an investigation into the relevance of using the prostaglandin H synthase (PHS) system for metabolising EDs. As described earlier, PHS activity is expressed in a wide range of tissues, and this includes hormonally-active organs, such as the uterus (e.g. by uterine stromal cells *in vitro* (Liu *et al.*, 2001)).

Lastly, it is most important that due consideration be given to the ability of indicator cell lines to conduct both phase I and II metabolism, to avoid the possibility of generating a large amount of false positive data due to the formation of hydroxylated metabolites that will possess structural features on the molecules that predispose them to binding to hormone receptors, without the possibility for these to be detoxified by conjugation.

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Table 1
EXAMPLES OF CELL LINES AND OTHER CELL TYPES USED IN CELL
PROLIFERATION AND TRANSCRIPTIONAL ACTIVATION ASSAYS FOR TESTING
ENDOCRINE DISRUPTORS (Combes, 2000a and references therein)

<i>Cell line</i>	<i>Estrogenicity</i>	<i>Androgenicity</i>	<i>Notes</i>
CHO	No	Yes	
CHO-K1	Yes	No	Metabolises vinclozolin
COS-1	Yes	No	Easy to transfect with recombinant CYPs
BG-1	Yes	No	
CV-1	No	Yes	Easy to transfect. Possesses 17 α - oxidase and 5 α - reductase
ELT-3	Yes	No	Also possesses PR receptor
HEC-1	Yes	No	Also possesses PR receptor
HEK 293	Yes	No	
Hep G2	Yes	Yes	Possesses some residual P450 activity and can be transfected with various CYPs, but has no ER α or β
Hep 1c1c7	Yes	Yes	Possesses Ah receptor
HeLa	Yes	No	
Ishikawa	Yes	No	Also possesses PR receptor
MCF-7	Yes	No	Also possesses PR receptor and metabolises a range of estrogens.
MDA-MB	Yes	Yes	Very low level of ER β ; GR receptor also present
PC-3	No	Yes	
PC-12	No	Yes	
PALM	No	Yes	
T47D	Yes	No	Also possesses PR receptor
ZR-75	Yes	No	Also possesses PR receptor
Yeast	Yes	Yes	Residual P450 activity present.

Table 2
SYSTEMS THAT CAN BE USED FOR THE TESTING OF METABOLISM IN VITRO: A TIERED APPROACH

Strategies	Test systems	Endpoints	Applicability	Formal Status
First tier	<p>Microsomes from human hepatocytes or from genetically engineered cell lines expressing human genes</p> <p>Cell lines, primary monolayer cultures, genetically engineered cell lines expressing human genes</p> <p>Microsomes, human hepatocytes, genetically engineered cell lines expressing human genes</p> <p>Microsomes, human hepatocytes, genetically engineered cell lines expressing human genes</p>	<p>Identification of metabolite formation by LC-MS/MS</p> <p>Cell morphology, viability, membrane damage, liver-specific endpoints, genotoxic endpoints</p> <p>Quantification by LC-MS of the amount of the parent compound that remains after metabolism</p> <p>Quantification by LC-MS, HPLC or fluorescence</p>	<p>Most important metabolic pathways</p> <p>Metabolism-mediated toxic effects</p> <p>Metabolic stability</p> <p>Inhibition</p>	<p>Prevalidation studies to be initiated</p>
Second tier	<p>Short-term and long-term hepatocyte cultures (e.g. human hepatocyte sandwich cultures), precision-cut liver slices and liver-derived cell lines expressing or re-expressing biotransformation enzymes, highly differentiated human cell lines</p>	<p>Assaying the activity of the biotransformation enzymes (and, if appropriate, their individual isoenzymes); quantifying the protein level by using techniques such as immunoblotting or HPLC; and quantifying the mRNA levels by using Northern blotting, the nuclease protection assay or the reverse transcriptase polymerase chain reaction</p>	<p>Induction</p>	<p>Prevalidation study to be initiated</p>
Third tier	<p>Genetically engineered cell lines expressing human genes</p>	<p>Quantification by LC-MS</p>	<p>Polymorphism</p>	<p>Prevalidation study to be initiated</p>

Table 3
SELECTED EXAMPLES OF METABOLISM OF STEROID HORMONES & EDs

Substrate(s)	System	Observation	Reference
Progesterone	Cytosolic and particulate NADPH and NADH 5 α -dihydroprogesterone-3 α -hydroxy steroid oxidoreductase with female rat brain fractions	Steroid metabolism shown to be present in neural cells	Li et al., 1997
Styrene oligomers	Rat liver microsomes from PB-induced rats with yeast and MCF-7 cell system	Activated to estrogenicity	Kitamura et al., 2003
Bisphenol A and B and methoxychlor	Same as above	Activated to estrogenicity	Yoshihara et al., 2001
2-nitrofluorene	Rat liver microsomes with MCF-7 assay	7-OH-NF formed and this was active whereas the parent compound was not.	Fujimoto et al. 2003
Testosterone	Recombinant V79Mz cell line stably co-expressing human CYP 3A4 and human NADPH oxidoreductase Adult and fetal human brain fractions	6 β - hydroxylation	Gebhardt et al., 1999
		Formation of 5 α -dihydroxytestosterone; 5 α -androstane-3 α ,17 β -diol and androstenedione due to 5 α -reductase and 17 β -hydroxysteroid dehydrogenase	Jenkins & Hall, 1977
Benzo[a]pyrene, chrysene, 2,2'-dichloro-, 4,4'-dichloro-, 2,2'-dibromo- and 4,4'-dibromo- biphenyl	Rat liver microsomes with induced CYP 1A1 and 1A2	Formation of estrogenic metabolites that bound to the ER, induced proliferation in T4D cells and in a reporter gene assay (ER-CALUX)	Meerman et al., 2003

Bisphenol A	Peroxidase with covalent binding to DNA exogenous system	Activation	Atkinson and Roy, 1995
Estrone	Liver microsomes from 14 human organ donors	All samples metabolised estrone with a 4-fold variation between individuals in efficiency. The 2-OH-metabolite was the predominant one, with the 4-OH and 16 α -OH metabolites also being formed	Shou et al., 1997
Progesterone	Recombinant yeast simultaneously expressing CYP17A1, CYP21B1 and yeast NADPH-P-450 reductase	Conversion to 17 α -hydroxyprogesterone and then further to 11-deoxycortisol	Sakaki et al., 1991
	Chimeric cDNA expression of P-450C5 and 2C4 in COS-1 cells	CYP 2C5 had 10-fold greater activity for generating progesterone 21-hydroxylation	Kronbach et al., 1989

Table 4.
CYP ISOZYME METABOLISM OF STEROID HORMONES & ENDOCRINE DISRUPTORS (Lewis, 1996)

CYP ISOZYME	SUBSTRATE	REACTION
1A1	Estradiol/Estrone	2- hydroxylation
	2-hydroxyestrone	2-hydroxylation
	Estradiol	14-hydroxylation
	4-hydroxyestrone	4-hydroxylation
	Testosterone	6 β -hydroxylation
1A2	Estrone	
	Testosterone	6 β -hydroxylation
2A1	Testosterone	6 α - and 7 α -hydroxylation
2A2	Testosterone	7 α -, 15 α -, 16 α -, 6 β - and 7 β - hydroxylation
3A1	Testosterone	6 β - hydroxylation
3A2	Testosterone	2 β - and 6 β - hydroxylation
3A4 (human)	Dexamethasone	6 α - and 6 β - hydroxylation
3A4 (rat)	Ortho-methoxychlor	Further hydroxylation
3A7	Testosterone	
3AhPCN3	Testosterone, progesterone and adrostenedione	6 β - hydroxylation
3AhPCN1	Testosterone, progesterone and adrostenedione	6 β - hydroxylation
11A1	Cholesterol	Formation of pregnenolone
17A1	Progesterone	17 α - hydroxylation
19A1	Androgens (e.g. adrostenedione)	19 hydroxylation to form estrogens via aromatisation (e.g. estrone)
21A1	Progesterone	21-hydroxylation
1B1	estradiol and estrone	16 α - and 16 β - hydroxylation
2B1	Testosterone	16 α -, 17 α - and 16 β - hydroxylation
2B2	Testosterone	16 α - and 16 β - hydroxylation
	16-hydroxyestrone	16 α - hydroxylation
2B6	Methoxychlor	Ortho-hydroxylation

11B1	Progesterone	11 β - hydroxylation (to corticosterone)
2C	Progesterone	16 α - and 6 β - hydroxylation
	Tamoxifen	4- hydroxylation
2C6	Testosterone	2 α - , 16 α - and 17 α - hydroxylation
2C7	Testosterone	16 α - hydroxylation
2C11	Testosterone	2 α - , 16 α - , 17 α - and 6 β - hydroxylation
	Cholesterol	11 α - hydroxylation
2C12	Cholesterol	12 α - hydroxylation
	Testosterone	6 β - hydroxylation
2C13	Testosterone	7 α - , 15 α - , 16 α - and 6 β -
2C19	Methoxychlor	Meta-hydroxylation (metabolised further by 3A4 and 2C19)
C17	Biosynthesis of androstenol	3 β - hydroxy dehydrogenase; 5 α - reductase; 17 α and 17,20- lyase
	Cholesterol	7 α - hydroxylation
C21	Cholesterol	21 α - hydroxylation
P-450	Steroids	C21- hydroxylation
4	Prostaglandins	ω - hydroxylation
7	Cholesterol	7 α - hydroxylation

Table 5

EXAMPLES OF TYPICAL ACTIVITY ASSAYS AND AVAILABILITY OF COMMERCIALY AVAILABLE ANTIBODIES IN ORDER TO ANALYSE XENOBIOTIC-METABOLISING CYP ENZYMES.

	<i>Activity Assay</i>	Commercial availability of antibody
CYP1A1	7-Ethoxyresorufin O-deethylase	Yes
CYP1A2	7-Methoxyresorufin O-demethylase; Phenacetin O	Yes
CYP1B1	7-Ethoxyresorufin O-deethylase; Phenacetin O-Deethylase	Yes
CYP2A6	Coumarin 7-Hydroxylase	Yes
CYP2B6	(S)-Mephenytoin N-Demethylase; Cyclophosphamid 4-hydroxylase Pentoxeresorufin O-depentylase Benzyloxyresorufin O-debenzylase	Yes
CYP2C9	Tolbutamidhydroxylase Diclofenac 4'-Hydroxylase	Yes
CYP2C19	Mephenytoin 4'-hydroxylase	Yes
CYP2D6	Debrisoquinhydroxylase Dextromethorphan Bufuralol 1'-hydroxylase	Yes
CYP2E1	Chlorzoxazon 6-hydroxylase	Yes
CYP3A4	Testosteron 6 β -hydroxylase Midazolam hydroxylase	Yes
CYP3A5	Testosteron 6 β -hydroxylase	Yes

Table 6 PROPOSED FRAMEWORK FOR THE TESTING OF ENDOCRINE DISRUPTORS AND METABOLISM
(N= Chemical is not active in the test; Y= Chemical is active in the test.)

Primary test for endocrine disruption <i>in vitro</i> (P)	Assessment of metabolism of the chemical <i>in vitro</i> (MC)	Assessment of metabolism of hormones <i>in vitro</i> (MH)	Test combining P and MC (PMC)	Test combining P and MH (PMH)	General <i>in vivo</i> testing (OECD 407, 415, 416) (G)	<i>In vivo</i> test with endpoints related primarily to endocrine disruption	Conclusions
N	N	N					Probably no ED
		Y			Y		ED
		Y				Y	ED
		Y			N	N	No ED*
	Y		N				Probably no ED
	Y		Y				Probably ED
	Y		Y		Y		ED
	Y		Y			Y	ED
	Y		Y		N	N	No ED*
Y			N				Probably no ED
Y			Y				Probably ED
Y			Y		Y		ED
Y			Y			Y	ED
Y			Y		N	N	No ED*
			Y				Probably ED
			Y		Y		ED
			Y			Y	ED
			Y		N	N	No ED*
			Y				Probably ED
			Y		Y		ED
			Y			Y	ED
			Y		N	N	No ED*

* If *in vitro* test in species used for *in vivo* test is positive

** It is not likely that this test would have been performed, but in the event that data exist a positive result should be treated similarly to a positive test result in a PMC test.

P450 Transcription Factors

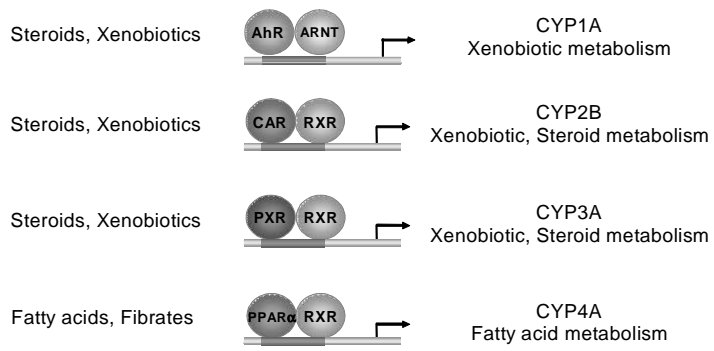


Figure 1. P450 Transcription factors (Jacobs, 2004)

Figure 2. Key receptors and P450 in the steroidogenic pathway (Jacobs, 2004)

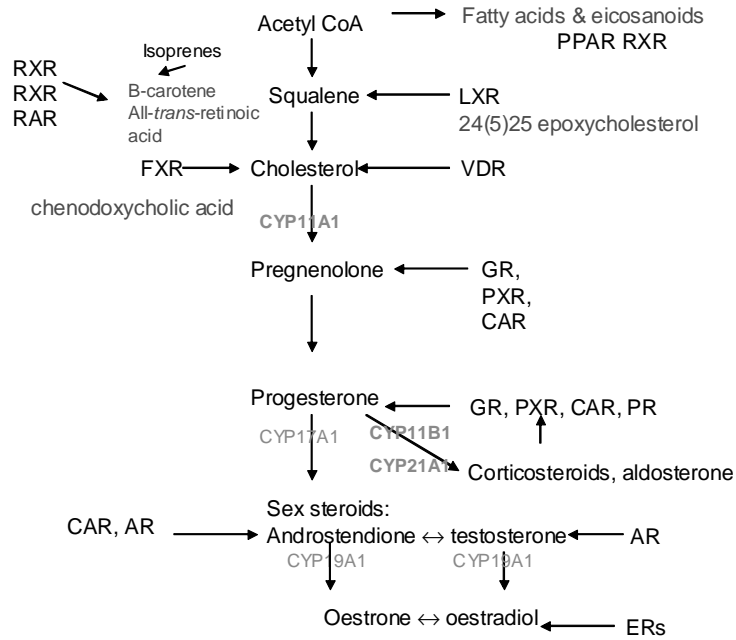


Figure 3. The synthesis of different steroid hormones with indications of the roles played by some P450s (Mensah-Nyagan et al., 1999)

