

Unclassified

ENV/JM/MONO(2008)24

Organisation de Coopération et de Développement Économiques
Organisation for Economic Co-operation and Development

29-Jul-2008

English - Or. English

**ENVIRONMENT DIRECTORATE
JOINT MEETING OF THE CHEMICALS COMMITTEE AND
THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY**

**SERIES ON TESTING AND ASSESMENT
Number 97**

**DETAILED REVIEW PAPER ON THE USE OF METABOLISING SYSTEMS FOR IN VITRO
TESTING OF ENDOCRINE DISRUPTORS**

JT03249306

Document complet disponible sur OLIS dans son format d'origine
Complete document available on OLIS in its original format



**ENV/JM/MONO(2008)24
Unclassified**

English - Or. English

ENV/JM/MONO(2008)24

OECD Environment, Health and Safety Publications

Series on Testing and Assessment

No. 97

DETAILED REVIEW PAPER ON THE USE OF METABOLISING SYSTEMS FOR *IN VITRO* TESTING OF ENDOCRINE DISRUPTORS

IOMC

INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

A cooperative agreement among UNEP, ILO, FAO, WHO, UNIDO, UNITAR and OECD

Environment Directorate

ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT

Paris 2008

Also published in the Series on Testing and Assessment:

- No. 1, *Guidance Document for the Development of OECD Guidelines for Testing of Chemicals (1993; reformatted 1995, revised 2006)*
- No. 2, *Detailed Review Paper on Biodegradability Testing (1995)*
- No. 3, *Guidance Document for Aquatic Effects Assessment (1995)*
- No. 4, *Report of the OECD Workshop on Environmental Hazard/Risk Assessment (1995)*
- No. 5, *Report of the SETAC/OECD Workshop on Avian Toxicity Testing (1996)*
- No. 6, *Report of the Final Ring-test of the Daphnia magna Reproduction Test (1997)*
- No. 7, *Guidance Document on Direct Phototransformation of Chemicals in Water (1997)*
- No. 8, *Report of the OECD Workshop on Sharing Information about New Industrial Chemicals Assessment (1997)*
- No. 9, *Guidance Document for the Conduct of Studies of Occupational Exposure to Pesticides during Agricultural Application (1997)*
- No. 10, *Report of the OECD Workshop on Statistical Analysis of Aquatic Toxicity Data (1998)*
- No. 11, *Detailed Review Paper on Aquatic Testing Methods for Pesticides and industrial Chemicals (1998)*
- No. 12, *Detailed Review Document on Classification Systems for Germ Cell Mutagenicity in OECD Member Countries (1998)*
- No. 13, *Detailed Review Document on Classification Systems for Sensitising Substances in OECD Member Countries 1998)*
- No. 14, *Detailed Review Document on Classification Systems for Eye Irritation/Corrosion in OECD Member Countries (1998)*
- No. 15, *Detailed Review Document on Classification Systems for Reproductive Toxicity in OECD Member Countries (1998)*
- No. 16, *Detailed Review Document on Classification Systems for Skin Irritation/Corrosion in OECD Member Countries (1998)*

- No. 17, *Environmental Exposure Assessment Strategies for Existing Industrial Chemicals in OECD Member Countries (1999)*
- No. 18, *Report of the OECD Workshop on Improving the Use of Monitoring Data in the Exposure Assessment of Industrial Chemicals (2000)*
- No. 19, *Guidance Document on the Recognition, Assessment and Use of Clinical Signs as Humane Endpoints for Experimental Animals used in Safety Evaluation (1999)*
- No. 20, *Revised Draft Guidance Document for Neurotoxicity Testing (2004)*
- No. 21, *Detailed Review Paper: Appraisal of Test Methods for Sex Hormone Disrupting Chemicals (2000)*
- No. 22, *Guidance Document for the Performance of Out-door Monolith Lysimeter Studies (2000)*
- No. 23, *Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures (2000)*
- No. 24, *Guidance Document on Acute Oral Toxicity Testing (2001)*
- No. 25, *Detailed Review Document on Hazard Classification Systems for Specifics Target Organ Systemic Toxicity Repeated Exposure in OECD Member Countries (2001)*
- No. 26, *Revised Analysis of Responses Received from Member Countries to the Questionnaire on Regulatory Acute Toxicity Data Needs (2001)*
- No. 27, *Guidance Document on the Use of the Harmonised System for the Classification of Chemicals which are Hazardous for the Aquatic Environment (2001)*
- No. 28, *Guidance Document for the Conduct of Skin Absorption Studies (2004)*
- No. 29, *Guidance Document on Transformation/Dissolution of Metals and Metal Compounds in Aqueous Media (2001)*
- No. 30, *Detailed Review Document on Hazard Classification Systems for Mixtures (2001)*
- No. 31, *Detailed Review Paper on Non-Genotoxic Carcinogens Detection: The Performance of In-Vitro Cell Transformation Assays (2007)*

- No. 32, *Guidance Notes for Analysis and Evaluation of Repeat-Dose Toxicity Studies (2000)*
- No. 33, *Harmonised Integrated Classification System for Human Health and Environmental Hazards of Chemical Substances and Mixtures (2001)*
- No. 34, *Guidance Document on the Development, Validation and Regulatory Acceptance of New and Updated Internationally Acceptable Test Methods in Hazard Assessment (2005)*
- No. 35, *Guidance notes for analysis and evaluation of chronic toxicity and carcinogenicity studies (2002)*
- No. 36, *Report of the OECD/UNEP Workshop on the use of Multimedia Models for estimating overall Environmental Persistence and long range Transport in the context of PBTS/POPS Assessment (2002)*
- No. 37, *Detailed Review Document on Classification Systems for Substances Which Pose an Aspiration Hazard (2002)*
- No. 38, *Detailed Background Review of the Uterotrophic Assay Summary of the Available Literature in Support of the Project of the OECD Task Force on Endocrine Disrupters Testing and Assessment (EDTA) to Standardise and Validate the Uterotrophic Assay (2003)*
- No. 39, *Guidance Document on Acute Inhalation Toxicity Testing (in preparation)*
- No. 40, *Detailed Review Document on Classification in OECD Member Countries of Substances and Mixtures Which Cause Respiratory Tract Irritation and Corrosion (2003)*
- No. 41, *Detailed Review Document on Classification in OECD Member Countries of Substances and Mixtures which in Contact with Water Release Toxic Gases (2003)*
- No. 42, *Guidance Document on Reporting Summary Information on Environmental, Occupational and Consumer Exposure (2003)*
- No. 43, *Guidance Document on Mammalian Reproductive Toxicity Testing and Assessment (2008)*
- No. 44, *Description of Selected Key Generic Terms Used in Chemical Hazard/Risk Assessment (2003)*
- No. 45, *Guidance Document on the Use of Multimedia Models for Estimating Overall Environmental Persistence and Long-range Transport (2004)*

- No. 46, *Detailed Review Paper on Amphibian Metamorphosis Assay for the Detection of Thyroid Active Substances (2004)*
- No. 47, *Detailed Review Paper on Fish Screening Assays for the Detection of Endocrine Active Substances (2004)*
- No. 48, *New Chemical Assessment Comparisons and Implications for Work Sharing (2004)*
- No. 49, *Report from the Expert Group on (Quantitative) Structure-Activity Relationships [(Q)SARs] on the Principles for the Validation of (Q)SARs (2004)*
- No. 50, *Report of the OECD/IPCS Workshop on Toxicogenomics (2005)*
- No. 51, *Approaches to Exposure Assessment in OECD Member Countries: Report from the Policy Dialogue on Exposure Assessment in June 2005 (2006)*
- No. 52, *Comparison of emission estimation methods used in Pollutant Release and Transfer Registers (PRTRs) and Emission Scenario Documents (ESDs): Case study of pulp and paper and textile sectors (2006)*
- No. 53, *Guidance Document on Simulated Freshwater Lentic Field Tests (Outdoor Microcosms and Mesocosms) (2006)*
- No. 54, *Current Approaches in the Statistical Analysis of Ecotoxicity Data: A Guidance to Application (2006)*
- No. 55, *Detailed Review Paper on Aquatic Arthropods in Life Cycle Toxicity Tests with an Emphasis on Developmental, Reproductive and Endocrine Disruptive Effects (2006)*
- No. 56, *Guidance Document on the Breakdown of Organic Matter in Litter Bags (2006)*
- No. 57, *Detailed Review Paper on Thyroid Hormone Disruption Assays (2006)*
- No. 58, *Report on the Regulatory Uses and Applications in OECD Member Countries of (Quantitative) Structure-Activity Relationship [(Q)SAR] Models in the Assessment of New and Existing Chemicals (2006)*
- No. 59, *Report of the Validation of the Updated Test Guideline 407: Repeat Dose 28-Day Oral Toxicity Study in Laboratory Rats (2006)*

- No. 60, *Report of the Initial Work Towards the Validation of the 21-Day Fish Screening Assay for the Detection of Endocrine Active Substances (Phase 1A) (2006)*
- No. 61, *Report of the Validation of the 21-Day Fish Screening Assay for the Detection of Endocrine Active Substances (Phase 1B) (2006)*
- No. 62, *Final OECD Report of the Initial Work Towards the Validation of the Rat Hershberger Assay: Phase-1, Androgenic Response to Testosterone Propionate, and Anti-Androgenic Effects of Flutamide (2006)*
- No. 63, *Guidance Document on the Definition of Residue (2006)*
- No. 64, *Guidance Document on Overview of Residue Chemistry Studies (2006)*
- No. 65, *OECD Report of the Initial Work Towards the Validation of the Rodent Uterotrophic Assay - Phase 1 (2006)*
- No. 66, *OECD Report of the Validation of the Rodent Uterotrophic Bioassay: Phase 2. Testing of Potent and Weak Oestrogen Agonists by Multiple Laboratories (2006)*
- No. 67, *Additional data supporting the Test Guideline on the Uterotrophic Bioassay in rodents (2007)*
- No. 68, *Summary Report of the Uterotrophic Bioassay Peer Review Panel, including Agreement of the Working Group of the National Coordinators of the Test Guidelines Programme on the follow up of this report (2006)*
- No. 69, *Guidance Document on the Validation of (Quantitative) Structure-Activity Relationship [(Q)SAR] Models (2007)*
- No. 70, *Report on the Preparation of GHS Implementation by the OECD Countries (2007)*
- No. 71, *Guidance Document on the Uterotrophic Bioassay - Procedure to Test for Antioestrogenicity (2007)*
- No. 72, *Guidance Document on Pesticide Residue Analytical Methods (2007)*
- No. 73, *Report of the Validation of the Rat Hershberger Assay: Phase 3: Coded Testing of Androgen Agonists, Androgen Antagonists and Negative Reference Chemicals by Multiple Laboratories. Surgical Castrate Model Protocol (2007)*
- No. 74, *Detailed Review Paper for Avian Two-generation Toxicity Testing (2007)*

No. 75, *Guidance Document on the Honey Bee (Apis Mellifera L.) Brood test Under Semi-field Conditions (2007)*

No. 76, *Final Report of the Validation of the Amphibian Metamorphosis Assay for the Detection of Thyroid Active Substances: Phase 1 - Optimisation of the Test Protocol (2007)*

No. 77, *Final Report of the Validation of the Amphibian Metamorphosis Assay: Phase 2 - Multi-chemical Interlaboratory Study (2007)*

No. 78, *Final Report of the Validation of the 21-day Fish Screening Assay for the Detection of Endocrine Active Substances. Phase 2: Testing Negative Substances (2007)*

No. 79, *Validation Report of the Full Life-cycle Test with the Harpacticoid Copepods Nitocra Spinipes and Amphiascus Tenuiremis and the Calanoid Copepod Acartia Tonsa - Phase 1 (2007)*

No. 80, *Guidance on Grouping of Chemicals (2007)*

No. 81, *Summary Report of the Validation Peer Review for the Updated Test Guideline 407, and Agreement of the Working Group of National Coordinators of the Test Guidelines Programme on the follow-up of this report (2007)*

No. 82, *Guidance Document on Amphibian Thyroid Histology (2007)*

No. 83, *Summary Report of the Peer Review Panel on the Stably Transfected Transcriptional Activation Assay for Detecting Estrogenic Activity of Chemicals, and Agreement of the Working Group of the National Coordinators of the Test Guidelines Programme on the Follow-up of this Report (2007)*

No. 84, *Report on the Workshop on the Application of the GHS Classification Criteria to HPV Chemicals, 5-6 July Bern Switzerland (2007)*

No. 85, *Report of the Validation Peer Review for the Hershberger Bioassay, and Agreement of the Working Group of the National Coordinators of the Test Guidelines Programme on the Follow-up of this Report (2007)*

No. 86, *Report of the OECD Validation of the Rodent Hershberger Bioassay: Phase 2: Testing of Androgen Agonists, Androgen Antagonists and a 5 α -Reductase Inhibitor in Dose Response Studies by Multiple Laboratories (2008)*

No. 87, *Report of the Ring Test and Statistical Analysis of Performance of the Guidance on Transformation/Dissolution of*

Metals and Metal Compounds in Aqueous Media (Transformation/Dissolution Protocol) (2008)

No.88 *Workshop on Integrated Approaches to Testing and Assessment (2008)*

No.89 *Retrospective Performance Assessment of the Test Guideline 426 on Developmental Neurotoxicity (2008)*

No.90 *Background Review Document on the Rodent Hershberger Bioassay (2008)*

No.91 *Report of the Validation of the Amphibian Metamorphosis Assay (Phase 3) (2008)*

No.92 *Report of the Validation Peer Review for the Amphibian Metamorphosis Assay and Agreement of the Working Group of the National Coordinators of the Test Guidelines Programme on the Follow-Up of this Report (2008)*

No.93 *Report of the Validation of an Enhancement of OECD TG 211: Daphnia Magna Reproduction Test (2008)*

No.94 *Report of the Validation Peer Review for the 21-Day Fish Endocrine Screening Assay and Agreement of the Working Group of the National Coordinators of the Test Guidelines Programme on the Follow-up of this Report (2008)*

No.95 *Detailed Review Paper on Fish Life-Cycle Tests (2008)*

No.96 *Guidance Document on Magnitude of Pesticide Residues in Processed Commodities (2008)*

No.97 *Detailed Review Paper on the use of Metabolising Systems for In Vitro Testing of Endocrine Disruptors (2008)*

© OECD 2008

Applications for permission to reproduce or translate all or part of this material should be made to: Head of Publications Service, OECD, 2 rue André-Pascal, 75775 Paris Cedex 16, France

About the OECD

The Organisation for Economic Co-operation and Development (OECD) is an intergovernmental organisation in which representatives of 30 industrialised countries in North America, Europe and the Asia and Pacific region, as well as the European Commission, meet to co-ordinate and harmonise policies, discuss issues of mutual concern, and work together to respond to international problems. Most of the OECD's work is carried out by more than 200 specialised committees and working groups composed of member country delegates. Observers from several countries with special status at the OECD, and from interested international organisations, attend many of the OECD's workshops and other meetings. Committees and working groups are served by the OECD Secretariat, located in Paris, France, which is organised into directorates and divisions.

The Environment, Health and Safety Division publishes free-of-charge documents in ten different series: **Testing and Assessment; Good Laboratory Practice and Compliance Monitoring; Pesticides and Biocides; Risk Management; Harmonisation of Regulatory Oversight in Biotechnology; Safety of Novel Foods and Feeds; Chemical Accidents; Pollutant Release and Transfer Registers; Emission Scenario Documents; and the Safety of Manufactured Nanomaterials.** More information about the Environment, Health and Safety Programme and EHS publications is available on the OECD's World Wide Web site (<http://www.oecd.org/ehs/>).

This publication was developed in the IOMC context. The contents do not necessarily reflect the views or stated policies of individual IOMC Participating Organizations.

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

This publication is available electronically, at no charge.

**For this and many other Environment,
Health and Safety publications, consult the OECD's
World Wide Web site (www.oecd.org/ehs/)**

or contact:

**OECD Environment Directorate,
Environment, Health and Safety Division**

**2 rue André-Pascal
75775 Paris Cedex 16
France**

Fax: (33-1) 44 30 61 80

E-mail: ehscont@oecd.org

FOREWORD

The background to this Detailed Review Paper (DRP) began at the meeting of the Validation Management Group for non animal testing (VMG-NA) in March 2003, where concerns were expressed that it was necessary to consider and preferably incorporate metabolism of compounds when considering the development of *in vitro* tests for endocrine active substances, to reflect the real *in vivo* situation, and so reduce the risks of false positives and false negatives. In response to this discussion, it was agreed that the development of a DRP would be necessary to address how metabolism is, or could be incorporated into *in vitro* test systems and give recommendations as to how to address this issue in future developments of such test methods.

In May 2003, at the 15th meeting of the Working Group of the National Coordinators of the Test Guidelines Programme (WNT), the Secretariat asked if a member country would be willing to take the lead for this project, and Belgium offered to do so. The project was then included in the rolling work plan. Preliminary texts provided by ICAPO and the European Commission (EC) were integrated and added to the document by Belgium.

A preliminary draft DRP was submitted by Belgium in November 2004 to the 2nd meeting of the VMG-NA and in January 2005 to the 8th EDTA Task Force meeting. The lead country and the Secretariat requested additional comments and further input. This was received from the European Commission, the Netherlands, and Germany, and the revised DRP was discussed by the 3rd meeting of the VMG-NA in December 2005. Following this meeting, further comments were received from Canada and Japan, and these were incorporated into the text, together with further editing and updating by the Secretariat. Also at this point, as the Belgium lead moved to a new position, the Secretariat (and then the European Commission) took the lead in completing the project.

In March 2006, the Secretariat presented the new draft DRP to the WNT requesting national expert reviews. In response to comments received from the United Kingdom, Denmark, the Netherlands, the United States and Germany, the text was pared down, extensively restructured, the focus sharpened and with this overhaul, in May 2006, the 18th WNT provisionally approved the document. Further constructive comments on this revision were received in response to requests from the Secretariat, from the United States and the United Kingdom prior to the 19th WNT in March 2007, and pending German national expert approval, the WNT approved the paper. Further comments were received from Germany at the end of April 2007, and these have been addressed and mostly incorporated into the text together with extensive editing and further updating by the European Commission.

Towards the end of 2007 and early 2008 additional comments were received from the United States. The comments were addressed and some further updates were also made where necessary. The draft DRP was approved by the WNT at its 20th meeting, in April 2008.

This document is published on the responsibility of the Joint Meeting of the Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology.

Many experts, including the National Coordinators, have participated in the discussions for this DRP during a number of different OECD meetings, and the Secretariat would especially like to mention:

- The main authors:

Miriam Jacobs, EC ECVAM

Walter Janssens, Scientific Institute of Public Health, Belgium

Robert Combes, ICAPO

Sandra Coecke, EC ECVAM

Esther Brandon, National Institute for Public Health and the Environment, the Netherlands

Ulrike Bernauer, Federal Institute for Risk Assessment, Germany;

- experts members of EC/ECVAM groups (Craig Mc Ardle, Alexius Freyberger, Bart van der Burg, Andreas Freidig, Ovanes Mekenyan)
- experts from the VMG-NA (Timothy Schrader, Masahiro Takeyoshi)
- experts that have been actively involved in the development, drafting and reviews of the document, particularly:

Dr Philippa Edwards, HPA, UK

Dr Karen Hamernik, US EPA

Dr Keith Houck, US EPA

Dr Patricia Schmieder, US EPA

Dr Richard Kolanczyk US EPA

Dr Alberto Protzel, US EPA

Mr. Gary Timm, US EPA

Dr Jerry Smrchek, US EPA,

Dr Abigail Jacobs, US FDA

Dr Calvin Willhite, Department of Toxic Substances Control, State of California, USA

TABLE OF CONTENTS

FOREWORD	12
ABBREVIATIONS USED:	16
EXECUTIVE SUMMARY	18
1. ENDOCRINE DISRUPTION	21
1.1 Introduction	21
1.2 Mode of action of EAS	22
1.3 The need for <i>in vitro</i> tests for EAS	24
1.4 <i>In vitro</i> tests for endocrine disruptors	24
1.4.1 In vitro receptor binding assays	24
1.4.2 In vitro functional tests: Induction of hormone-responsive mammalian cell proliferation	25
1.4.3 In vitro functional tests: Transactivation assays (hormone-sensitive transcription of reporter genes)	25
2. METABOLISM	27
2.1 The role of metabolism in the activation and detoxification of xenobiotics: Phase I and Phase II metabolism	27
2.2 Use of S9 systems in <i>in vitro</i> testing for mutagenicity	28
2.3. Special considerations for <i>in vitro</i> systems testing for endocrine activity	28
2.3.1 Induction of biotransformation enzymes	29
2.3.2 Models for evaluating polymorphic effects on metabolism	30
2.3.3. Methoxychlor, a case study of the influence of metabolism on endocrine activity	30
2.4 Measurement of metabolism	33
2.4.1 Current status of computer-based approaches for assessing metabolism	33
2.4.2 In vitro systems	35
2.4.2.1 Cell-free systems	36
2.4.2.2 Purified enzymes	36
2.4.2.3 Cytosolic soluble enzyme fractions (S100 supernatant) and microsomal particulate enzyme fractions (S100 pellet)	37
2.4.2.4. Post-mitochondrial S9 supernatant (consisting of both cytosol and microsomes).....	37
2.4.3 Intact cells	37
2.4.4. In vivo considerations	39
2.5 Potential problems with using exogenous metabolism in EAS studies	39
2.6 Foetal and infant metabolism	41
2.7 Metabolism during digestion	41
3. THE METABOLISM OF ENDOGENOUS STEROIDS	43
3.1 Introduction	43
3.2 Metabolic activation of endogenous steroids	44
3.3 Metabolic inactivation of endogenous steroids	45
3.4. Dermal metabolism	45
3.5 The consequences of metabolism for hormonal activity	46
4. THE TESTING OF ENDOCRINE DISRUPTORS AND METABOLISM	47
4.1 Introduction	47
4.2 Possible ways to overcome problems of using exogenous systems	48

4.2.1	The intrinsic capacity of cell lines used for testing EAS	48
4.2.2	The potential to use genetically-engineered cell lines	49
5.	CONCLUSIONS AND RECOMMENDATIONS	50
5.1.	Proposals to develop a framework for the simultaneous testing of endocrine disruption and metabolism .	51
5.2.	Approaches to combine the <i>in vitro</i> study of endocrine disruption and metabolism	51
5.2.1.	Short term approaches	51
5.2.2.	Medium term goals	52
5.2.3.	Long term goals	53
6.	REFERENCES	55
7.	TABLES	80
8.	FIGURES	92

ABBREVIATIONS USED:

AhR: Aryl hydrocarbon Receptor
AR: Androgen Receptor
ARNT: AhR Nuclear Translocator
BPA: Bisphenol A
CAR: Constitutive Androstane Receptor
CAT: Chloramfenicol Transferase
CERI: Chemicals Evaluation Research Institute, Japan
CYP: cytochrome P450
DNA: Deoxyribonucleic acid
DRE: Dioxin Response Element
DRP: Detailed Review Paper
EA: Endocrine Activity
EAS: Endocrine Active Substances
ED: Endocrine Disruptor
ER: Estrogen Receptor
ECVAM: European Centre for the Validation of Alternative Methods
EDTA: OECD Task force for Endocrine Disruptor Testing and Assessment
EDSTAC: Endocrine Disruptor Screening and Testing Advisory Committee
EPA: Environmental Protection Agency (U.S.)
FSH: Follicle-Stimulating Hormone
GFP: Green Fluorescent Protein
GR: Glucocorticoid Receptor
hEST: human Estrogen Sulphotransferase
HTP: High Throughput
HRE: Hormone Response Element
HPLC: High Performance or High Pressure Liquid Chromatography
JaCVAM: Japanese Centre for the Validation of Alternative Methods
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
METI: Ministry of Economy, Trade and Industry, Japan
MHLW: Ministry of Health, Labour and Welfare, Japan
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
P450: cytochrome P450
PCB: Polychlorinated Biphenyl
PBDE: Polybrominated Diphenyl Ether

PHS: Prostaglandin H Synthase system
PR: Progesterone Receptor
PXR: Pregnane X Receptor (also termed SXR: Steroid and Xenobiotic Receptor)
(Q)SAR: (Quantitative) Structure Activity Relationship
(Q)SPR: (Quantitative) Structure Property Relationship
RT PCR: Reverse Transcriptase Polymerase Chain Reaction
SPSF: Short Project Submission Form
SULT: Sulphotransferase
VMG-NA: OECD Validation Management Group: Non Animal
WNT: OECD Working Group of National Coordinators of the Test Guidelines Programme

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. Chemicals found to test positive are considered to be endocrine active substances (EAS) and may be putative 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 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 EAS. This situation is in sharp contrast to genotoxicity testing, where *in vitro* tests are routinely conducted with and without metabolic capacity. This Detailed Review Paper aims to justify why *in vitro* assays for EAS should incorporate metabolising or metabolic enzyme systems, and to indicate how this could be done. Tests to detect EAS 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 still being developed. Therefore it is not possible at present to incorporate measurements of metabolism into *in vitro* testing for endocrine disruption within the context of validated tests. However, the eventual need to combine the outcome of these developments will be an important component of the development of each field. Information derived from existing tests that take metabolism into account could perhaps already be used to prioritise models for further development.

The background to EAS/ED testing, the available test methods, and the role of mammalian metabolism in the activation and the inactivation of both endogenous and exogenous steroids are described. The available types of systems are compared, and the potential problems in incorporating systems in *in vitro* tests for EAS, and how these might be overcome, are discussed. Lastly, some recommendations for future activities are made.

EAS 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 EAS 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 work to overcome some of these potential problems, to optimise yeast assays, have been recently published. There is also a wealth of EAS/ED data generated by these systems in the public domain that, with normalisation, might be usefully incorporated into multivariate and (Quantitative) Structure Activity Relationships ((Q)SAR) EAS/ED analyses. It is important to determine the intrinsic metabolic capacity of both mammalian and yeast cells already used in proliferation and transfection assays, for endocrine activity (EA). It might be also be worthwhile to investigate the potential to incorporate metabolic capacity into these cell types.

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, including skin, small intestine, colon and placenta. Metabolising systems for use in *in vitro* EAS assays can be either intact organs and tissues, subcellular (isolate/purified enzymes, cell homogenates, cytosol, microsomes, S9) or cellular (the inherent metabolism of the indicator cells, metabolically competent primary cells or cell lines or genetically-engineered cell lines). Despite the fact that there are several technical problems with using sub-cellular systems in assays for testing EAS (such as non-specific binding to protein, and the potential toxicity of the subcellular fraction to the intact cells in the assay system following insertion), 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 EAS.

The use of (Q)SAR and (Quantitative) Structural Property Relationships ((Q)SPR) to indicate chemicals that could be metabolised to derivatives with altered endocrine activity or interfere with metabolism of endogenous and exogenous substances should be developed further. *In vitro* or *in vivo* methods could also be used to determine metabolites formed. Both parent compounds and metabolites predicted by *in silico*, *in vitro* or *in vivo* methods could then be tested in the *in vitro* model for endocrine activity. However, great care would need to be taken to optimise and streamline such an approach, otherwise it is likely to be slow and costly.

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 or acceleration of endogenous hormone metabolism, such as via the steroidogenic pathway and drug metabolising pathways, 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). As yet, there is relatively little information on the intrinsic metabolic potential of cell systems traditionally used to detect endocrine activity. It is recommended that this information is obtained as a matter of urgency. 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 EAS activity. Examples of such cell constructs are known to be currently under development by at least two different research groups and show promise. Following these developments, feasibility studies on 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 enzymes should be initiated. 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 utilising added subcellular fractions. Furthermore, metabolites formed may not need to be transported from their sites of formation to intracellular targets. If these developments are confirmed, they should be developed into tests that can be used for regulatory purposes.

Investigation into the relevance of using the prostaglandin H synthase (PHS) system and other, non-cytochrome P450 dependent enzymes, for metabolism of EAS is also required.

Much could be learned from genotoxicity testing, where metabolism is already incorporated. One possibility to make quick progress is to incorporate an S9 fraction into the *in vitro* tests for endocrine disruption. However, the potential toxicity that S9 could cause to target cells, limitations arising from the complexity of the endocrine system, as compared to mutagenicity test models, and the problem of potential protein binding, need to be carefully addressed and well controlled.

It is concluded that the perceived difficulties of incorporating metabolism into *in vitro* tests for EAS should not deter more research into how this can be achieved. The development and validation of these *in vitro* assays in conjunction with appropriate systems should be a matter of urgency. 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 may ultimately need to be combined. No *in vitro* system will mirror the complexity of metabolism in the whole animal, but this should not prevent the use of *in vitro* systems, provided the limitations are clearly understood and the results interpreted appropriately.

1. ENDOCRINE DISRUPTION

1.1 Introduction

Exposure to natural and synthetic chemicals in the environment, with potential to interfere with endocrine systems, may elicit a 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, though not universally 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.

Recently, interest has focused on exogenous substances that disturb sex steroid and thyroid hormone function, because most evidence for ED activity has been produced in these domains, and tests have been made available that focus on these targets (EDSTAC 1998). However, other steroid hormone systems may be similarly susceptible to disturbance by xenobiotic substances. The function of peptide hormones such as follicle stimulating hormone (FSH), luteinizing hormone (LH), insulin, insulin like growth factors, growth hormone etc., may also be affected by exogenous chemicals, but it is likely that the characteristics will differ from those found for steroid hormones. Although it may be advisable to explore these possibilities, it is premature to include them in a screening strategy with regulatory implications. This Detailed Review Paper (DRP) will therefore focus attention on the hormones for which there is currently evidence that they are affected by xenobiotics. Mechanistic screening studies, whether *in vivo* or *in vitro*, are used to detect effects on a specified endocrine function. To reflect this, the term endocrine active substances (EAS) will be used in this paper to cover all mechanistic tests, and the term endocrine disruption is reserved for adverse health effects secondary to such activity.

The mechanisms that may be involved, and that are covered by this DRP include:

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

This list is not exhaustive. Potential mechanisms not considered in this DRP include those involving neurotoxicant or neuroendocrine targets and certain aspects of metabolism, particularly those involving esterases that may be affected by cholinesterase inhibitors.

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 and discussing legislation for ED testing (e.g. see Anon, 2001b). The European Commission will reassess the latest developments in scientific knowledge with respect to substances identified as having endocrine disrupting properties by June 2013. On the basis of that review the Commission may, if appropriate,

present legislative proposals (Article 138: L 396/142 EN Official Journal of the European Union 30.12.2006).

In the USA, the EPA was charged with validating assays and screening pesticide chemicals to detect those that may affect human health by mimicking oestrogen. For this purpose, the EPA established an Endocrine Disruptors Screening and Testing Advisory Committee (EDSTAC) to consider possible testing strategies. EDSTAC recommended broadening the screening programme to include the androgen and thyroid hormone systems and to include effects on wildlife. 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 and Leydig cell cultures. 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: [<http://www.epa.gov/scipoly/oscpendo/edspoverview/finalrpt.htm>].

An OECD Working Group on Endocrine Disruptor Testing and Assessment (EDTA) has been established to direct and oversee the development of guidelines for the detection of ED substances as part of the Test Guidelines Programme. 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 EAS, namely, a uterotrophic assay for estrogenic activity, the Hershberger test for androgenic activity, and an enhanced protocol for repeat dose toxicity to detect ED agents acting on a range of hormonal systems, including thyroid and sex hormone systems. The development, standardisation and validation of *in vitro* tests to detect endocrine disrupting activity is in progress, and although interference of chemicals with steroidogenic enzymes and aromatase may be a major pathway towards ED activity, this will not be dealt with here in detail as they are already the subjects of separate DRPs at the OECD.

The possibilities for *in vitro* testing of metabolism in a more general context and with regard to cosmetics have been previously addressed by ECVAM. These efforts generated much information that is also relevant to endocrine disruption testing *in vitro*, and has stimulated the development of a research programme to develop the field further.

The aim therefore of the present DRP is to review the available evidence on the need for inclusion of mammalian metabolic competence into the *in vitro* EAS/ED test system designs and to suggest possibilities to meet this need, without duplicating the work that is already being done in the development and validation of tests for endocrine disruption under the auspices of the OECD, or for kinetics and metabolism of chemicals (Coecke, et al., 2004; Coecke, et al., 2006), but rather to bring these two approaches together.

While non-mammalian and ecotoxicological considerations are highly relevant to the use of systems for *in vitro* testing of EAS, these aspects are not addressed directly here, as they are more appropriate for a separate DRP. Furthermore, it is also not the intention of this DRP to address all the factors which influence the interpretation of information derived from *in vitro* tests, to predict potential risks to humans.

1.2 Mode of action of EAS

EAS act by a diversity of mechanisms including receptor binding, altered post-receptor activation, altered hormone synthesis, perturbation of hormone storage, release, clearance and homeostasis. The immediate

effects of these activities are to enhance or suppress the action of the endogenous hormones, including changes in homeostatic and feedback mechanisms.

This DRP focuses particularly on the estrogen and androgen hormone systems. In this context, the term estrogenic means that a chemical has the same effect as 17 β -estradiol acting at the estrogen receptor (ER) in a particular system. Anti-estrogenic means that a substance prevents 17 β -estradiol, or another recognised natural or synthetic agonist, acting at the ER, from exerting its effect in a particular model. Similarly, the term androgenic means that a chemical has the same effect as testosterone, acting at the androgen receptor (AR) in a particular system. Anti-androgenic means that a substance prevents testosterone, or another recognised natural or synthetic agonist acting at the AR, from exerting its effect in a particular model.

The effects are mediated in part by binding specifically to one or more of an extensive family of nuclear receptors (Warner, et al., 1999) that trigger or suppress the transcription of a specific set of genes (Hornhardt, and Wiebel, 1996; Wiegel, 1996; Combes, 2000; Nilsson, et al., 2001; Jacobs, and Lewis, 2002).

The nuclear estrogen receptors (ERs) exist as at least 2 subtypes, termed α and β , encoded by distinct genes (Orti, et al., 1992; Graham, and Clarke, 1997; Kuiper, et al., 1998; McLachlan, 2001; Nilsson, et al., 2001) and with different tissue distribution, relative ligand binding affinities and biological functions (reviewed in Jacobs, 2005). There has been identification of a γ subtype in teleosts that may mediate some effects of EAS in these fish (Hawkins, et al., 2000; Hawkins, and Thomas, 2004). Furthermore, there is an ER 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, it can also be activated by larger molecules unable to enter the nucleus and bind with the nuclear ERs (Harrington, et al., 2005). Most studies have been directed to the sub-form of the nuclear ER, and therefore combined assessments of steroid metabolism and interference with estrogenic mechanisms should include this subtype. Nuclear ER α mediates the 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). Models currently being developed to measure estrogen receptor binding and activation mainly relate to ER α . Consideration of the adequacy of addressing only ER α binding for regulatory screening purposes is beyond the scope of this paper, but the requirements for metabolising systems may be similar irrespective of whether the test system is designed to determine effects upon the ER α , ER β or other steroid receptors.

The interest in thyroid hormones has arisen because they play a role in the control of development (e.g. metamorphosis in amphibians and brain development in mammals) (Yen, 2001), and a number of chemicals that alter their synthesis, transport and catabolism have been identified (Lazar, et al., 1993; Fowles, et al., 1994; Capen, 1997; Cheek, et al., 1999; DeVito, et al., 1999; Kato, et al., 2000; Zhou, et al., 2001; Craft, et al., 2002; Howdeshell, 2002; Schmutzer, et al., 2007). Interference by xenobiotics with thyroid hormone turnover 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 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.

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) and progesterone (Kuil, et al., 1998). Thus, a mouse hepatoma cell line (HepalC1c7) possessing this receptor, in conjunction with a Dioxin Response Element (DRE) 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, appears to have a fundamental role in cellular physiology, neurodevelopment and circadian rhythmicity (Poellinger, 2000). Ligand binding results in interaction with the DRE on the DNA, resulting in transcription of a group of enzymes active in steroid

and xenobiotic metabolism (Hahn, 1998; 2002). Such a complex mechanism may be extremely difficult to investigate *in vitro*, but a simple AhR assay may be useful to detect chemicals that need further investigation with respect to interference with metabolism. Similarly, other nuclear receptors including for example the Pregnane X Receptor (PXR) (Moore, et al., 2000) and the Constitutive Androstane Receptor (CAR) (Wei, 2000) are involved in modulating the effects of endogenous and exogenous steroids and xenobiotics metabolising CYPs (CYP2B and CYP3A respectively), as illustrated in figures 1-3, and also drug transporters in human cells, but they do not influence the basal expression levels (Friedman, et al., 2002).

In the case of polypeptide hormones utilising intracellular signaling pathways, agents that interfere with these pathways may prove to be EAS, and there may be other modes of action to be considered in the future, as our knowledge base increases.

1.3 The need for *in vitro* tests for EAS

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. 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 prioritising chemicals for further testing, and also to facilitate the interpretation of data from animal tests. As such, it is crucial that false negative results are minimised. A certain level of false positive results is acceptable, although should be low, given that a false positive result is likely to lead to animal testing with the resultant financial and animal costs.

From an animal welfare point of view, potentially very large numbers of animals will be required for ED testing, should the current EPA proposals go ahead. The actual numbers will depend on the sensitivity and specificity of the testing methods to be used (Purchase, 1999). The use of animals is 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 EAS.

1.4 *In vitro* tests for endocrine disruptors

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 inhibit the binding of standard ligands (see Häggblad, et al., 1995). Theoretically they should allow high throughput (HTP) screening technology to be used. However it may be impractical to conduct extensive metabolic profiling of all chemicals before they are tested in HTP endocrine screening assays. Nevertheless, it may be useful to develop and use some general metabolic activation systems, combined with reporter gene expression, to enhance the probability that the endocrine screening assays will detect a chemical's endocrine disruption potential. Competitive ligand binding assays, whether alone or combined with reporter gene expression, are designed to detect chemicals that directly interact with the purified receptor proteins. (Gray, et al., 1997). With regard to the ERs, the subtype that is being investigated is mostly the α -subtype. The AR and thyroid receptors have been proposed for inclusion into the screening for endocrine disruptors by EDSTAC and inclusion of ER β should also be considered. Ligand binding studies cannot distinguish between agonists and antagonists; functional *in vitro* tests are therefore required for this purpose. Measuring the recruitment of coregulator proteins to nuclear receptors in response to ligand binding provides a functional *in vitro* binding assay capable of detecting agonists, partial agonists, and antagonists (Chen, et al., 2003). A variety of assay

formats have been used including AlphaScreen (Rouleau, et al., 2003), fluorescence resonance energy transfer (Liu, et al., 2003), and fluorescence polarisation (Ozers, et al., 2005). These assays can be very sensitive as well as high-throughput. In addition, use of cytometric bead arrays for coregulator recruitment permits multiplexing of assays. Multiplexed binding assays enable the exploration of complex binding interactions in a single reaction volume. Individual fluorescent microsphere populations allow separate molecules to be coupled to their surface such that multiple nuclear receptor-coactivator protein binding interactions can be investigated (Iannone, et al., 2001). Inclusion of metabolic capacity in the assay, e.g. S9 mix, has not been reported for these assay formats and would require determination of potential for signal interference.

Such assays are very attractive from a screening standpoint because they are:

- a. Very sensitive
- b. Can detect both agonists and antagonists
- c. Can potentially be multiplexed
- d. Have very high-throughput and can thus test many chemicals rapidly and relatively inexpensively
- e. May have less sensitivity to the presence of S9 mix (no information of this is available, however)
- f. Lack a cell membrane barrier so metabolites from S9 mix incubation for example would have ready access to the target.

1.4.2 In vitro functional tests: 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 (Ramamoorthy, et al., 1997; Fang, et al., 2000; Soto, and Sonnenschein, 2001). 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 also has intrinsic metabolic capacity, the potential advantages may outweigh the possible shortcomings. Catalogues indicating the metabolic competence of cell lines are available (Hornhardt, & Wiebel, 1996; Tew, et al., 1996), but updating would be very useful.

If metabolism is included in functional tests, one should realise 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 (Kupfer, and Dehal, 1996; Kauffman, et al., 1997; Jordan, and Morrow, 1999; Dutertre, and Smith, 2000; Liu, et al., 2003) depending on species, tissue and receptor examined. Such substances therefore provide valuable tools for exploration and validation of the predictions of tests under development.

1.4.3 In vitro functional tests: 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; Ramamoorthy, et al., 1997; Fang, et al., 2000). Examples are given in Table 1.

The cells are transformed (or transfected) by introducing vectors bearing DNA sequences for human or mammalian nuclear ERs, in conjunction with the appropriate response elements, promoter region and reporter gene. Some potential problems with yeast-based systems include: effects on assay system of test materials that have fungicidal activity or inhibit cell proliferation, solubility, permeability or transport issues due to such things as presence of a cell wall (ICCVAM, 2003), molecular size, or molecular structure, and the potential confounding activity of endogenously produced metabolites or the failure of the yeast system to metabolise test material (Nakano, et al., 2002). Work to overcome some of these potential problems, to optimise yeast assays has been recently published. A strain of genetically modified yeast (*Saccharomyces cerevisiae*) cells have been modified with respect to deletion of drug resistance transporters (Sievernich, et al., 2004; Hasenbrink, et al., 2006), 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 differentiation between agonists and antagonists (Kohno, et al., 1994; Dudley, et al., 2000; Legler, et al., 2002a; ICCVAM, 2003). This problem can be overcome 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 (LUMI-CELL® ER Assay). Similarly 293 HEK cells can be transfected with ER β receptors (Meerts, et al., 2001; Lemmen, et al., 2002). Transactivation assays have also been generated that make use of endogenous receptors and inserted reporter genes, such as human breast cancer-derived cell lines (Demirpence, et al., 1992; Miller, et al., 2000; Freyberger, and Schmuck, 2005) and ER CALUX (Legler, et al., 1999). Interestingly, ER CALUX cells have been used in combination with S9 (Legler et al. 2002a, b).

Further work in the area of the use of metabolising systems for *in vitro* testing of endocrine disruptors is warranted. For those cell-based systems used to characterise the endocrine activity of chemicals, the metabolic competency should be assessed. This may provide some idea of whether these systems are likely to metabolise chemicals, and what aspects of metabolism may need to be supplemented.

2. METABOLISM

2.1 The role of metabolism in the activation and detoxification of xenobiotics: Phase I and Phase II 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) (Lewis, 1996; Xu, et al., 2005). The key enzymes for phase I oxidation are the isoforms of the CYP family of enzymes. Regulated by nuclear receptors (Honkakoshi, and Negishi, 2000), 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. Figure 1 indicates the principal NRs that induce key P450 transcription factors for phase I metabolism of steroidal and xenobiotic substances. While the liver is the main site of xenobiotic metabolism, it is important to note that phase I and II metabolism also occurs in most tissues and the gut microflora (Combes, 1992; Ding, & Kaminsky, 2003; Nishimura, et al., 2003; Furukawa, et al. 2004; Coecke, et al., 2006), and may differ from hepatic metabolism (Bernauer, et al., 1999; 2000; 2002; 2003a). Usually, these conversions result in a decrease in toxicity and/or an increased excretion of the chemical. However, metabolic activation is also possible, which can also be inhibited or induced by pharmaceutical, environmental and dietary chemicals (e.g. Lewis, 1996; Michnovicz, et al., 1997; Lampe, et al., 2000; Rose, et al., 2000; Nakajima, et al., 2001; Lanza, et al., 2004; Sheets, et al., 2004).

In contrast to the drug-metabolising CYPs, the CYPs that catalyse steroid and bile acid synthesis have very specific substrate preferences (See figures 2 and 3) (You, 2004). For example, the CYP that catalyses the formation of estrogen from testosterone, CYP19 (aromatase), only metabolises testosterone and does not metabolise xenobiotics (Lewis., 1996; Goodman and Gilman., 2006). However it is a potential target for environmental chemicals and a steroidogenesis assay, using the H295R cell line which expresses most of the important steroidogenic enzymes, CYP11A, CYP11B, CYP17, CYP19, and CYP21, is currently undergoing prevalidation as one of the Tier 1 Screening battery Alternate Methods.

The finding that metabolism by certain CYPs may show stereo specificity, and the likelihood that at least for a number of chemicals the interaction with the target receptor may also show stereo specificity, suggests that particular attention will need to be paid to the testing of chemicals with chiral centers. The carbon position regioselectivity and stereo selectivity in steroid metabolism by cytochrome P450s (Waxman, 1988; Brocks, 2006) therefore also needs to be a subject for consideration in the development and utilisation of *in vitro* assays linked to, or including metabolic enzyme systems.

The key phase II enzymes include N-acetyl transferases (NAT), UDP-dependent glucuronosyl transferase (UGT), sulphotransferases (SULT), 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 trap 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 prior phase I metabolism. This has been reported for instance for aromatic hydroxyl groups in beta-adrenergic agonists (Smith, 1998) and for halogenated compounds (reviewed by Guengerich, 2005). Sulphatases, phase II cleavage enzymes can also be involved in steroid metabolism forming O sulphates.

While these general considerations on the influence of metabolism on toxicity are not specific to endocrine disruptors, they are particularly important to this field for the reasons outlined in section 2.3 below.

2.2 Use of S9 systems in *in vitro* testing for mutagenicity

No *in vitro* system will mirror the complexity of *in vivo* metabolism, and the production of active metabolites may be over or underestimated. However, these considerations should not prevent the use of systems, provided the drawbacks are clearly understood and the results used in an intelligent fashion. The evolution of genotoxicity testing offers a good parallel for the use of a metabolising system to improve the relevance of an *in vitro* test. In the case of Ames testing (OECD 1997), a metabolizing system in the form of S9, containing microsomal and cytosolic fractions prepared from rat liver (usually) pretreated with Arochlor 1254, to induce metabolising enzymes, has been built into the tests. Appropriate alternatives to Arochlor1254 are a combination of phenobarbitone and β -naphthoflavone (Elliot et al., 1992). The latter combination conforms with the Stockholm Convention on Persistent Organic Pollutants (UNEP 2004) and has been shown to be as effective as Arochlor 1254 for inducing mixed-function oxidases (MFOs). The S9 fraction typically is used at concentrations ranging from 1-10% (v/v) in the final test medium. The condition of metabolic activation system may depend upon the the class of chemical being tested and in some cases it may be appropriate to utilize more than one S9 concentration. Over the years these tests have been proved to be useful and are widely accepted.

The test is accepted because, in part, it is used as a non-quantitative screening assay for a non-threshold activity. This may be less acceptable in a test where information on the dose-response and thresholds for activity may be more important. For mutagenicity, it is also a more effective strategy because a mutation, once generated, is irreversible and accumulates during the test.

2.3. Special considerations for *in vitro* systems testing for endocrine activity

Metabolism is a particularly important consideration when testing EAS, since several hormonally-active chemicals, including some naturally-occurring EAS, are known to be subject to metabolism (Table 1). It is known that metabolism of natural steroids can alter their biological activity. For example, 16 α -hydroxyestrone is generated from 17 β -estradiol in MCF-7 cells (Miyamoto, & Klein., 1998). While the former is still estrogenic, it is also capable of damaging DNA, and may influence the cell cycle and proliferative responses of MCF-7cells (Lewis, et al., 2001; 2005). The sulphation, cleavage, and recycling of estrogens and thyroid hormones by SULT enzymes and sulphatases is a particularly important route for the removal of active hormones from the body. Whilst most of the information on metabolism has been derived from studies with endogenous steroids, exogenous chemicals structurally related to endogenous steroids may be expected to undergo analogous transformation reactions (Nishiyama, et al., 2002).

Xenobiotics may also exert effects attributable to an effect on endogenous steroid metabolism. An older literature example of this are the extensive alterations of 17 β oestradiol metabolism reported in response to environmental chemicals such as 2,3,7,8, tetra-chlorodibenzo-p-dioxin (2, 3, 7, 8-TCDD) (Spink, et al., 1990). More recently, as part of the series of European Commission funded research programmes, *in vitro* assays utilising 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, (Kirk, et al., 2003; Davies, et al., 2004; Harris, et al., 2005; 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.

Such indirect effects must be borne in mind in the design of models for testing for steroid hormone activity and interpretation of results, although for practical purposes inclusion of tests for indirect action in the same models is unlikely, but a battery approach is possible.

There are also many examples in the scientific literature of intra and extracellular transporters impacting upon the availability of chemical substances (especially drugs and steroids) within the cell. These are therefore also another important consideration, particularly the expression of efflux pump ATP binding cassette (ABC) transporters such as multiple drug resistance efflux pumps (e.g. MDR1) and P-glycoprotein (Pgp) which can limit intracellular exposure. As for drug metabolism, strategies to assess EAS metabolism and transporter interactions potential are necessary (Tucker et al., 2001), and should be included in a testing framework for EAS metabolism screening.

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 EAS and that need to be taken into account when developing tests that will measure metabolism and endocrine effects *in vitro*. A review of methoxychlor activity is therefore included as section 2.3.3 below. 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. Luciferase based transfection assays such as ER CALUX (Legler, et al., 2002a, b) can also use a combination of S9 and give a measure of estrogenic activity. Provided that such tests can be made sufficiently specific, they may offer an alternative testing method (Rogers, & Denison., 2000).

There are many examples in the scientific literature of metabolism converting the endocrine activity of a range of substances. Some examples are given in Table 2. This suggests that inclusion of metabolism is needed in EAS testing to increase the sensitivity of the test and to augment the reliability to predict the effect of a chemical. In the long term it might also become an advantage for improved hazard characterisation and risk assessment if one could predict the potency of the mixture of parent compound and metabolites to which sensitive targets are exposed. 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 or EAS.

2.3.1 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 (RT PCR) (Coecke, et al., 1999).

Although there are technical difficulties associated with the cryopreservation of hepatocytes, a number of these 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 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 & 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-nonachlor 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 EAS that interact with these receptors and that otherwise would not have been detected.

2.3.2 *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 (Lin, and Lu, 2001), which can lead to serious toxic side-effects (Wolf, & Smith, 1999) and consequences for the risk assessment process (Bernauer, et al., 2003a).

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 EAS mechanisms is improving (Burchell, et al., 1998; 2000; Eaton, & Bammler, 1999; Glatt, 2000; MacGregor, et al., 2001; Nishiyama, et al., 2002; Glatt, et al., 2005). Although genetic polymorphisms of metabolising enzymes are considered to be potentially important for endocrine disruption because they may make a group of people more or less susceptible to the enzymes' action, they are not a high priority with respect to establishing the possible hazards that chemicals have to induce hormonal effects. Genetic polymorphism biomarkers are probably more important for epidemiological biomonitoring.

2.3.3 *Methoxychlor, a case study of the influence of metabolism on endocrine activity.*

The actions of methoxychlor and its metabolites provide a good example of the types of influence metabolism can have on endocrine activity and so are reviewed here. Methoxychlor (bis[*p*-methoxydichlorophenyl]trichloroethane - M) 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). Methoxychlor undergoes sequential de-methylation by CYPs (mostly 2C19 and 1A2) yielding mono-demethylated then bis-demethylated metabolites (see Fig. 4). The resulting bis-demethylated (also termed di-hydroxylated) metabolite of methoxychlor, 2,2-bis(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE), is estrogenic and anti-androgenic *in vitro* and its formation *in vivo* can explain the estrogenicity and anti-androgenicity of methoxychlor (Gaido, et al., 1999). Both monodemethylated and bis-demethylated methoxychlor are hydroxylated at the ortho position on the ring to yield phenolic groups by CYPs 3A4 and 2B (Stresser, & Kupfer, 1997, 1998a; 1998b; Dehal, & Kupfer., 1999) regulated by both PXR and CAR (Lehmann, et al., 1998; Waxman, 1999; Goodwin, et al., 2002).

CYP3A4 does not cause direct hydroxylation of methoxychlor (Stresser, & Kupfer, 1998b). This observation means that a prosubstrate (methoxychlor) is turned by one CYP (2C19 or 1A2) into a substrate (monodemethylated or bisdemethylated methoxychlor) for another CYP (3A4) (Stresser, & 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, & 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, & Kupfer, 2000a; 2000b). CYP2B6 has, however, very narrow substrate specificity (Hu, & Kupfer, 2000b).

HPTE has estrogenic activity and is more potent and has higher efficacy than the parent compound (Bulger, et al., 1978; Shelby, et al., 1996; Beresford, et al., 2000). HPTE also inhibited seminiferous cord formation *in vitro* much more than the parent compound methoxychlor (Cupp, & 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 HPTE is a more potent estrogen agonist than mono-demethylated methoxychlor that in turn is more potent than methoxychlor (Kupfer, & 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-demethylated metabolites of this product are also estrogens (Kupfer, & Bulger, 1987).

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). 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 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.

The methoxychlor metabolites: bis-, tris-hydroxy methoxychlor and a catechol, 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). (Supersomes are microsomes prepared from insect cells infected by baculovirus and containing cDNA of a single human CYP isoenzyme). 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 that the system is able to detect the 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, see figure 4 (Hu and Kupfer, 2002a). It is known that induction of enzymes including many CYPs and phase II metabolism enzymes can occur through NRs, in particular CAR and PXR (Lehmann, et al., 1998; Handschin, & Meyer, 2003) but also the glucocorticoid receptor (GR) and the peroxisome proliferator-activated receptor α (PPAR α) (see figure 1) (Hines, et al., 2001, reviewed in Jacobs, 2005). In rats exposed to methoxychlor for four 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. However this finding may reinforce the idea that considering testing for thyroxine metabolism and thyroid receptor activation may indeed be worthwhile at some stage of EAS and ED screening.

Methoxychlor and its metabolites, including mono- bis- and tri-hydroxylated compounds, bind covalently to hepatic microsomal proteins, and this is catalysed by CYPs (Bulger, & 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 postulated (but disputed) relationship between AhR and ER (Ohtake, et al., 2003; Shipley & Waxman, 2006), 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 explanatory component 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.

2.4 Measurement of metabolism

One approach to the investigation of the influence of metabolism on the endocrine activity of a chemical is to look at these two events sequentially, rather than trying to set up a model which combines both. This can be done by a prior evaluation of the effects of metabolism using at least four levels of capability, instead of merely *in vitro* versus *in vivo* systems. Such methods include *in silico*, *in vitro* or *in vivo* methods, and then the results of these tests can be used subsequently to determine which metabolites should be tested alongside the parent compound in subsequent *in vitro* tests for endocrine activity.

So when looking at metabolising systems it may be more useful to consider:

- (1) *In silico* methods: computational metabolite prediction, (Q)SAR and receptor/P450/SULT and other enzyme molecular modelling docking studies;
- (2) *in vitro*-subcellular systems - where cofactors are required to be added and phase I and II may not necessarily be present together dependent upon exactly what cofactors are added (e.g., microsomes, cytosol, isolated enzymes)
- (3) *in vitro* self-contained systems – no additional cofactors needed (e.g., cells, tissue slices)
- (4) *in vivo* - can be very different dependent upon time, dose, and what tissue, fluid, excreta is used for metabolite assessment.

2.4.1 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., 1997; 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 simulators have been developed for predicting biodegradation (CATABOL (Jaworska, et al., 2002)) and liver tissue metabolism (TIMES (Mekenyan, et al., 2004a)) The tissue metabolism simulator 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 prediction tools to facilitate the prediction of metabolic activation of chemicals in integrated systems; thus, the TIMES system has been used to predict mutagenicity and skin sensitisation whilst also accounting for the metabolism of chemicals (Mekenyan et al. 2004a; 2004b).

However recent peer evaluations of such expert systems in the fields of reproductive toxicity and skin irritation have found them to be of limited predictive value compared to *in vitro* systems (Maslankiewicz, et al., 2005).

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).

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 a single QSAR for EAS an unrealistic goal. However specific QSARs with broad applicability domains (AD) for the potential of a xenobiotic to be activated or inactivated by metabolism, or to affect metabolism of endogenous hormones should be achievable. However so far, particularly with respect to the European chemical inventories for high and low production volume chemicals (European chemical substances Information system (ESIS) (<http://ecb.jrc.it/esis>) and the European Inventory of Existing Commercial Chemical Substances (EINECS), chemical structures are available for approximately 50% of each inventory (Netzeva, et al., 2006).. In the US, the US EPA have recently initiated an international chemical prioritisation research project (ToxCast) that aims to evaluate chemical properties and effects, including ED effects across a wide range of information domains. There is another ongoing effort concerning predictive QSARs in developmental and reproductive toxicity evaluations that include the endpoints commonly considered in ED bioassays. This effort is funded in large part by Health Canada following an earlier analysis (Julien, et al., 2004). The QSAR database is being developed with the assistance of Leadscope Inc. (Columbus, Ohio). As of April 2006, volunteers from United Kingdom and U.S. academia, the U.S. FDA, NIEHS and EPA, state governments, contract research laboratories and major pharmaceutical firms are entering *in vivo* ED/EAS and other developmental toxicity data from the published literature. It is anticipated that proprietary data will also be entered as the project progresses.

Additionally, ECVAM (Jacobs, et al., report of expert workshop in preparation) are exploring *in silico* molecular modelling tools for EAS metabolism for regulatory purposes, further. Molecular dynamics are useful for revealing facets of activation and inactivation, so improving mechanistic understanding (Jacobs, et al., 2003; 2004; Vedani, et al., 2005). However objective assessment of CYP and receptor protein-ligand docking performance for internal validation and regulatory validation purposes are necessary.

Associative libraries can be utilised to give corrected predictions. Such an approach is useful for the modifications and updating of models before needing to rebuild, and this is an important consideration in the validation of QSARs for hazard and risk assessment regulatory purposes.

Strategically, molecular modelling and (Q)SAR techniques have value in the first tiers of integrated testing strategies for processing chemicals. However as identified here, the practical limitations of these *in silico* tools with respect to regulation of chemicals needs to be acknowledged, to provide realistic expectations of such methods.

2.4.2 *In vitro* 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, immortalised primary cells, liver-derived cell lines re-expressing biotransformation enzymes and genetically-engineered cell lines expressing biotransformation enzymes (Doehmer, 1993; Schmalix, et al., 1996; Houston, and Carlile, 1997; Clarke, 1998; Philip, et al., 1999; Coecke, et al., 1999; 2000; 2001; Bull, et al., 2001; Brandon, et al., 2003). In order to identify the most important metabolic pathways (including resistance of a substance to metabolism), 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 any metabolites by HPLC or GC would be more time consuming than the application of modern approaches like LC/MS/MS. If metabolites can be identified and isolated, or synthesized in sufficient quantities, they could be tested in *in vitro* assays that aim to detect endocrine activity (Freyberger, & Scholz, 2004). At present this approach may not be possible for large scale screening, although automation of metabolic stability studies in microsomes, cytosol and plasma (Linget, and Vignaud., 1999) and P450 inhibition (Miller, et al., 2000) have been reported. With the former, 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. Differences can be found depending upon the *in vitro* subcellular model used. For instance, Tang, et al., 2005, noted that some metabolites found in cytosol and in S9 were not found in microsomes for the compound they studied. Others have also noted the importance of using intact cells or slices that are metabolically competent (Lopez-Garcia, et al., 2005), as many cell lines are known to have lost this metabolic competence capacity over time (Castell, et al., 2005), and the addition of cofactors may affect the outcome of the assay (Dean, et al., 2004). Fish liver slices, for example, overcome such problems as they allow the detection of both metabolic capability and ER-mediated response in the same assay. (Shilling, & Williams, 2000; Schmieder, et al., 2000; 2004).

The advantage of cell-free exogenous systems is that the enzymes are in direct contact with the xenobiotics. However, unless the indicator endocrine 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 indicator cells to exert their effect. As a major target for EAS 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, and metabolites leave these cells and enter the

indicator cells or interact with targets located in their membranes. In cells genetically-engineered to metabolise xenobiotics and detect endocrine activity, the xenobiotic still has to penetrate the cell, but metabolites are generated intracellularly. The location of the target and site of metabolite formation may be important issues to consider. The transport through the cell may need particular attention if transport proteins are present in the *in vitro* system utilised (discussed in section 2.3). It should be born in mind that the need for metabolites to travel between cells is often the situation *in vivo*, where the major site of metabolism is other than the target organ.

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 the OECD and 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 large species differences in toxicokinetics; this is especially true for the metabolic differences between humans and rodents (Ozawa, et al., 2000). Therefore, human-based *in vitro* models may offer predictions of potential hazard to humans that would not 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 3.

2.4.2.1 Cell-free systems

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

- purified enzymes
- cytosolic soluble enzyme fractions (S100 supernatant) and 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 and belong in tier 2 of the summary of currently available approaches as given in Table 3.

Since no *in vitro* metabolic system will fully represent all potential *in vivo* metabolism, a negative result for a particular chemical in an *in vitro* assay with a metabolic system will probably not be a definitive negative for that chemical for all metabolic pathways.

2.4.2.2 Purified enzymes

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).

Recommendations to study the feasibility of using purified P450 and other enzymes in genetically engineered mammalian cell lines is considered technically challenging, but emerging research and development technologies are available. Under contract to Exxon Mobil, CXR Biosciences for example have utilised a GAL4 system for individual P450s (Elcombe, pers. comm; reported at the NHR DevTox [a specific support action of the EC sixth framework programme] workshops, April 2005). Recombinant

human CYPs can also be utilised from recombinant bacterial expression systems (bactosomes) to identify CYP metabolism pathways (Stanley, et al., 2005).

2.4.2.3 Cytosolic soluble enzyme fractions (S100 supernatant) and microsomal particulate enzyme fractions (S100 pellet)

Cell extracts can be fractionated by several centrifugation steps. At 100000 x g for 1 h the P100 pellet and the S100 supernatant are produced. Proteins associated with larger organelles (e.g. mitochondria, vacuoles, endoplasmic reticulum) are found in S9-S13 pellets, while proteins associated with the golgi or vesicles are found in S100 pellets. The upper, slower migrating membrane bound proteins tend to be found in the pellet, whereas the lower, faster migrating band (observed by Western blotting), are mainly found in the S100 supernatant, which contains the soluble proteins. These fractions contain many of the phase II enzymes.

Kupfer and Bulger, (1987) have used rat liver microsomes and NADPH as the system in the presence of immature rat uteri ER as the bioassay for the endocrine activity. Human hepatic microsomes have also been used in combination with the yeast cell assay to measure the estrogenic activity of methoxychlor (Elsby, et al., 2001a).

2.4.2.4. Post-mitochondrial S9 supernatant (consisting of both cytosol and microsomes)

It has been shown 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.

The use of S9 in combination with an assay for endocrine disruption testing may have a number of shortcomings. In cases where a non-human 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 if a source of human S9 would be available in sufficient quantities for screening, the potential variability remains 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 EAS testing is a likely development for the near future.

There is some literature that demonstrates the possible usefulness of a combination of S9 and ED targets (see sections 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 EAS 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.

2.4.3 Intact cells

Cell systems that can be used in *in vitro* assays fall into three main categories: a) inherent metabolism of the hormone activity indicator cells; b) co-culture systems of the indicator cells with a metabolically-competent cell line (e.g. freshly isolated hepatocytes or genetically-engineered cell lines); and c) genetically-engineered cell lines that simultaneously act as both indicators of endocrine activity 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 activity data (Combes, 1992). The level and specificity of metabolism in currently available indicator cells is unknown although it is usually assumed to be low. Investigation of the intrinsic metabolism in the indicator cells forms an important part of understanding the development of any test system and could be considered part of the validation of a proposed method. Indicator cells with adequate intrinsic metabolic capacity would be the most useful model, and it may be possible to refine the test to enhance the metabolic activity, to make it more reproducible and predictable. When metabolising systems (cellular co-culture or subcellular fractions) are added, the residual metabolism in the target cell type may become less important because it would be overwhelmed by the added metabolic capacity.

Fully competent cells like hepatocytes are not yet useful for screening purposes (with respect to reproducibility) on a large scale, and in particular if human hepatocytes are required (see Blaauboer, 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 yet possible to grow hepatocytes in culture under stable conditions to allow screening of large series of chemicals, although stem cell techniques developed for drug discovery could potentially be developed for such purposes in the longer term (Gebhardt, et al., 2003; Hengstler, et al., 2005; Hewitt, et al., 2007). 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. At the recent ECVAM workshop, the use of human hepatocytes was not considered to be possible at present for screening on a large scale (Coecke, et al., 2006).

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.

An additional approach of this type would involve the transient transfection cells with a cocktail of enzyme expression plasmids; it may be possible to create cells expressing appropriate metabolic enzymes, perhaps in relevant ratios. A nuclear receptor reporter gene could be included in this mix or the cell line could stably express it. Current transfection technologies are very efficient and result in limited cytotoxicity. Variability between transfections can be controlled by large-scale batch transfections followed by cryopreservation of cells in an "assay-ready" format. However the simultaneous expression of various enzymes in one cell line (or the ratio of gene products formed in the culture of the respective cell lines) should be interpreted with caution as clearly they may not reflect a real-life-situation. For example, in an established cell line which expresses human CYP2E1 and human sulphotransferase 1A1, extremely high CYP2E1 activities have been observed (Glatt, et al., 2005).

Viral transduction systems could also be considered using a cocktail of viruses to generate the metabolic activity desired. 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), so multiple cell lines will need to be created. This approach

would benefit from use of a stable cell line expressing the reporter gene system with transient transfection of only the P450 genes in order to reduce variability in response between the different CYP assays.

A further consideration when choosing a 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. However, the HepG2 human hepatoma cell line transiently transfected with the human AR and an androgen-responsive reporter (Maness, et al., 1998) was about 10 times less responsive to methoxychlor than HPTE, the more active androgen antagonist. As identified in section 2.3.3, these particular assay conditions appear to have affected the cells metabolic capacity to convert methoxychlor into its metabolite. This raises the question as to whether hepatocytes with metabolic competency and containing the relevant receptors can be constructed to produce a more useful screening assay.

In addition to the systems already discussed, there is significant effort underway in developing liver microreactors for use in metabolism and toxicity testing. Such three-dimensional, perfused microtissue units have been shown to maintain primary rat liver cells closer to the *in vivo* state than other culture methods (Sivaraman, et al., 2005; Schmitmeier, et al., 2006). Use of engineering approaches to this technology may provide systems with throughput sufficient to meet the needs of an ED/EAS testing programme.

2.4.4. *In vivo* considerations

Finally, while it is clear that any exogenous metabolising systems include Phase I and Phase II activities, other factors such as excretion also contribute to the fate of a compound. Phase I and Phase II reactions *in vivo* take place in an open system. Thus *in vivo*, a compound that becomes conjugated might be inactivated by excretion. Whereas, *in vitro*, the conjugated compound may still be toxicologically active if the pharmacophore has not been destroyed. Additional factors include selective binding, transport and distribution into target tissues (Ito, et al., 1997). These various factors should be considered in the incorporation of metabolic thinking into *in vitro* ED testing. A challenge is how one might model the effect of excretion and the downstream consequences of phase II conjugations.

The effect of different enzymes in a cascade may be quite different from the sum of effects of individual enzymes, and metabolism may not even 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 enzymes would need to be incorporated into cells if one uses genetically engineered cells for testing. It may not be easy to replicate *in vivo* metabolism with genetically engineered cells designed to express the enzymes involved. To date however, it is possible to express many different CYPs in the same cells, maintain expression levels, and keep them in culture (e.g. Mak, et al., 1999, Sanderson, et al., 2001, Stanley, et al., 2005, and references in section 1.4.3). Therefore a battery approach based upon *in vivo* mechanistic knowledge with simultaneous or sequential use of different cell lines each expressing different xenobiotic P450 enzymes, may be a constructive approach.

2.5 Potential problems with using exogenous metabolism in EAS studies

The paucity of published studies involving the use of exogenous metabolism in *in vitro* assays for EAS is surprising. It is recognised that there could be difficulties when adding exogenous 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 EAS and/or their conjugated metabolites 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 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 EAS and metabolites might be an important determinant of its *in vivo* activity, as well as influencing results from *in vitro* studies.

A study on 64 chemicals performed in a reporter gene assay making use of S9 indicated an additional problem 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, the standard reference agonist, was inactivated at normally active concentrations, 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. (Takeyoshi, M Pers. Comm).

The logistical problems described above of developing exogenous metabolising systems for combination with *in vitro* assays under development for detecting endocrine activity are not trivial. A recently published report (ICCVAM, 2003) on the use of *in vitro* binding and transcriptional activation (TA) assays for EE, concluded 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.*' (ICCVAM, 2003). Since this report was published, however, a number of binding and TA assays have been selected and are undergoing validation by US, European and Japanese validation bodies, under the auspices of the OECD (Table 4). It is therefore appropriate that metabolic activation systems are designed for these tests in particular.

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 estrogenic *in vitro*. *In vivo*, a number of these will be further metabolised and no longer be active. As a consequence, the use of 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.

In addition to considering physiologic concentrations of candidate EDs when designing *in vitro* metabolism protocols, it is also important to consider the forms of the delivered (target tissue) dose. Taking Bisphenol A (BPA), an estrogenic component of plastics and epoxy resins, as an example, this substance exists almost exclusively *in vivo* as the glucuronide (with small amounts of the sulfate) bound to plasma protein (~95%) (Kurebayashi et al., 2003). Measurement of tissue BPA levels is fraught with problems (e.g., possible laboratory contamination, stability of the glucuronide and sulfate metabolites, homogenisation of whole tissue without correction for the presence of the glucuronide in plasma) including reproducibility of the different analytical methods used (Fukata et al., 2006). These problems can result in overestimation of physiological BPA levels by 150-200 fold those actually present (Tominaga et al., 2006). In addition to knowing whether the BPA-glucuronide (like the BPA-sulfate) can or cannot enter the cells *in vitro* (Suiko et al., 2000; Snyder et al., 2000), it is equally important to know whether the pharmacologically-active entity (be it parent compound or metabolite) can transit from the circulation to the putative target tissue(s).

Given the problems identified here, a practical approach might be to use the knowledge about metabolites formed *in vivo* to guide the design of *in vitro* systems, even to the extent of testing known metabolites. Indeed, this may be a better route to follow than adding systems such as S9.

2.6. Foetal and infant metabolism

Phase I oxidative enzymes and phase II enzymes are expressed differently in foetal life and during later stages (Hines, & McGarver, 2002; McGarver, & Hines, 2002). Thus the metabolism of xenobiotics and the synthesis and degradation of steroids may differ in the developing foetus, due to both foetal metabolism and as a consequence of placental biotransformation. As the foetus may be particularly sensitive to the effect of EAS (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 EAS with the CYP family in the foetus may be needed. There is experimental evidence using a combination of CYP apoprotein (Western blot analysis, immunohistochemistry) and mRNA (Northern Blot, RT-PCR) for the presence of CYP1A1, CYP2C8, CYP2D6, CYP2E1, CYP3A4, CYP3A5, CYP 4A1 and particularly CYP1B1 and CYP3A7 in the foetal liver (Hakkola, et al., 1998; de Wildt, et al., 1999).

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 foetal 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 foetal liver, whereas glutathione S-transferases and UDP glucuronosyl transferases develop within 3 months after birth (Cresteel, 1998).

Therefore, in general, metabolism to more active substances in foetal tissues *in vivo* is unlikely to be missed in a system containing metabolising capacity derived from adult animals. Given also that foetal exposure is preceded by maternal exposure, it is unlikely that *in vitro* systems based on adult metabolism will result in significant false positive or negative results. The insertion of foetally specific relevant P450s in the design of the *in vitro* systems indicated in sections 2.2 and 2.3 could potentially address future regulatory requirements in this area.

2.7. Metabolism during digestion

The diet represents a major route of exposure to possible EAS (e.g., Wilson et al., 2003, Wilson et al., 2007), 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 emphasised 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 (Challis, 1973; Tannenbaum, et al., 1975; Spiegelhalter, et al., 1976; Assembly of Life Sciences., 1981, Chapters 4 and 6). The phenolic structure of several EAS 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 BPA, 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). Reductive metabolism by bacteria occurs in the intestines. This 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 β (Setchell, et al., 2005) and PXR (Jacobs, et al., 2005), although it is metabolised far more rapidly than synthetic EAS, which are often more persistent. At present, no systems that would mimic these types of

reactions are being considered for inclusion in tests for endocrine activity. It is likely that derivatives that are predicted or known to be produced would need to be tested in addition to the parent compound.

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 3) 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 figures 2 and 3). 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, & Lewis, 2002; Lewis 2003), 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 3). CYP3A4 is known to be able to hydroxylate estradiol (Stresser, & Kupfer, 1997). This enzyme also plays a prominent role in human metabolism of xenobiotic chemicals and drugs. Clearly 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 oestrinol. Estrone is further hydroxylated at the 2-, 4- or 16- positions by different CYPs (Table 3). 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 3). 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, & 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. Age related modulation of CYP enzyme activity is 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), and are discussed in section 2.3.2.

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 (hEST), (as discussed in section 2) thereby maintaining the available estrogen at higher levels (Kester, et al., 2000, 2002; Shevtsov, et al., 2003; Harris, et al., 2005). Other Detailed Review Papers are dealing in much more detail with the enzymes involved in the synthesis of steroids and aromatases. It is however important to realise 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, and this should be taken into account in test development.

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, and Table 5 for selected examples of metabolism of steroid hormones and EAS). 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.

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.

It is not known whether certain enzymes are specifically expressed in tissues like uterus, brain, testes, ovary and breast at a specific stage in life and may thus be affected differentially by some EAS. The breast is one of the tissues where local expression of CYP enzymes is described (Stromstedt, et al., 1993; Hellmold, et al., 1995, 1998a,b; Warner, et al., 1997, 1998), which may be relevant to endocrine disruption because of its sensitivity to estrogens.

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.

Conjugation may not necessarily inactivate, there are many examples of Phase II (conjugate) bioactivation in mammals (Miller, et al., 1968; Irving, 1971; King, & Glowinski, 1983; Wolf, et al., 1984; Anders, et al., 1986; Elmarakby, 1995; Glatt, et al., 2001; Altunas, et al., 2003; Dekant, 2003; Ghaoui, et al., 2003; Ross, & Pegram, 2004; Rietjens, et al., 2005). An example of a deleterious glucuronidation is glucuronidation of a carboxy group, yielding a reactive acyl glucuronide.

3.4 Dermal metabolism

Other extrahepatic tissues that may be important to consider are the skin and adipose tissue, due to the potential contribution to both systemic metabolism and dermal exposure via skin absorption, of xenobiotics and steroids (Janjua, et al., 2007). The skin is particularly active in the metabolism of 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 summarised 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 are expressed in the epidermis or in cultured keratinocytes. Although there is the potential for skin metabolism to modify systemic hormonal actions of dermally applied substances, firm evidence that this is a significant phenomenon *in vivo*, with respect to EAS/EDs, is lacking.

3.5 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

Section 2 described the role of metabolism and introduced a range of *in silico/in vitro* methods that have potential in the measurement of metabolism. 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, and these are described here.

A human microsomal 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 BPA, 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 they 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) has 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 has also shown that dibrominated biphenyls are bioactivated by CYPs to metabolites with estrogenic activity and inhibiting 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 BPA 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). In addition it was shown that in humans, BPA may be rapidly excreted as glucuronidated metabolites (Völkel, et al., 2005).

This indicates that species differences need to be addressed and the physiological relevance of using rat or human systems should be established.

However if the goal of the assay is to be for use as a screening tool such that qualitatively a (+) or (-) is sufficient with respect to EA activity, a good correlation of EAS yes/no activity across species has been shown, although absolute potency may vary. Olsen, et al., 2005 compared fish and mammal ER binding for a limited number of compounds. In all cases when some degree of binding was noted for one species, it was also for the other. While the magnitude of binding will vary across species, the yes/ no evaluation will be similar for both. Since there is no standardised method used across investigators and species, any apparent differences observed may also be due to test conditions differences as to inherent species differences. The same scenario holds true for many more chemicals as evidenced by the ER literature for various species, and comparing data for the same chemical.

Testing for metabolism of the test substance to more or less endocrine active substances may require approaches different from those required to detect interference of the test substance with metabolism of endogenous hormones.

Further, 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 systems

The presence of proteins that not only may bind steroids, but possibly also exogenous EAS, may hamper the use of cell free systems or cells with metabolic capacity, in conjunction with indicator cells that contain the endocrine target. This issue should be addressed carefully during the standardisation and validation procedure. However, it should be noted that this has not prevented the widespread use of S9 preparations in the Ames test. It may, after all, be advisable to incorporate a mandatory *in vitro* test to measure 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 use cells that 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. Such systems have the drawback that the tests cannot be carried out in the presence and absence of the metabolising system. In the case of genetically engineered cells, care would need to be taken to ensure that the metabolic capacity does not greatly exceed the *in vivo* situation, such that substances rapidly metabolised to inactive derivatives might be missed.

4.2.1 The intrinsic capacity of cell lines used for testing EAS

Little is known about the intrinsic metabolic competence of the usual mammalian cell lines that are used for detecting EAS acting via receptor binding. In this context, it is of interest that discrepancies in the

estrogenicity of methoxychlor (see Section 2.3.3) 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. MCF-7 cells are capable of generating the same spectrum of hydroxylated metabolites of estrone as are found *in vivo* (Bradlow, et al., 1995) although there may be quantitative differences.

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).

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 active 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) (Johansson, et al., 2002), and is currently in (pre)validation under the lead of the US EPA. This cell line expresses CYPs 1A1, 11A, 17, 19, 21, 1B1 and 11B1, which are differentially induced by EAS (Sanderson, et al., 2001).

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 II 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 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 identified at a recent ECVAM workshop is the limited number of CYPs that can be incorporated into one cell line. At present it is difficult or even impossible to study the impact of a chemical upon a chain of enzymes where the reaction product of the one is the substrate for the other, as was demonstrated for methoxychlor (see section 2.3.3), in a single genetically engineered cell line. 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. CONCLUSIONS AND RECOMMENDATIONS

Clearly, it is crucially important that *in vitro* methods for testing for endocrine activity should take account of metabolism, either in the form of subcellular enzyme fractions, or by using cells that have been rendered metabolically competent. Endogenous steroids and structurally related substances are extensively metabolised by phase I and II enzymes in both the liver and hormonally-active tissues, and such metabolism can lead to the activation, inactivation or changed activity. Despite the fact that there are several technical problems with using sub-cellular systems in assays for testing EAS, there is sufficient evidence in the literature to show that these limitations can be overcome, and that the presence of such enzymes modulates the endocrine activity of several different types of EAS.

The absence of metabolism in *in vitro* assays for endocrine activity could give rise to false positive data (due to lack of detoxification), or false negative data (lack of activation), arising from the use of the tests. The incorporation of metabolism into endocrine activity testing can contribute data on the potency of the metabolites of the parent compound, relevant to the possible duration of action. Such development requires careful consideration so as not to generate an unreasonable level of false positives and false negatives.

A potential limitation with chemical unknowns will be general applicability questions for the many techniques mentioned herein. For example, the *in vitro* genotox assays (with metabolic capabilities added) did not have a problem-free or necessarily quick development, and predictivity for potential mammalian chemical carcinogenicity has limitations.

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

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

The development and validation of methods to specifically detect endocrine disruption are ongoing at the OECD, and it is anticipated that tests will become available that can take into account metabolism and endocrine targets simultaneously.

Evidently, any information obtained from *in vivo* testing could also be used in a final framework for the combined *in vitro* testing of EAS and 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 have been recently peer reviewed, but it may also be obtained from other validated toxicity assays.

5.2. Approaches to combine the *in vitro* study of endocrine disruption and metabolism

At present there are no as yet validated *in vitro* tests incorporating metabolic competence to measure the effect of chemicals on endocrine targets *in vitro*.

A major point with regard to the development of metabolising systems for use *in vitro* is metabolic competence compared with the mix of diverse enzymes that is important *in vivo*. Ideally, the balance between activation and deactivation of compounds that may exist *in vivo*, should be preserved.

5.2.1. Short term approaches

In spite of the shortcomings described herein, the incorporation of S9 mix presents an approach that can be recommended for immediate investigation, and may be the only reasonable approach that is available now. Systems incorporating S9 mix should be investigated and plans developed to critically evaluate these systems for their applicability as well as their limitations.

In the short term, the quickest and easiest way to implement a combined test may be to incorporate S9 into acceptable indicator tests for endocrine activity, in a fashion analogous to that used in testing for mutagenicity. However there are many different types of S9 preparations. Constituent (activity can be low) and induced enzyme fractions can come from a number of different species and tissues; although human liver microsomes and/or S-9 preparations are mentioned, there may be difficulties in the viability, stability, activity, and standardisation of these preparations. Specifically, possible problems like protein binding, incubation time with S9, cytotoxicity of S9, and substances used to induce enzymes in the S9, should be investigated, but should not preclude the development of appropriate protocols. Although there are several reports in the literature about the successful use of such systems, they are very limited and perhaps notable for their uniqueness.

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 risk assessment for humans is the ultimate goal, then a human based system may be preferred, assuming the tissues from whence they came could be legitimately, ethically and transparently obtained.

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 problems of availability and standardisation 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 excessive rates of false positive results, which would lead to unnecessary further testing, should also be avoided. Metabolic capacity *in vitro*, in excess of that *in vivo*, may result in the unrealistically high degradation of the xenobiotic in the *in vitro* test and consequently reduced

sensitivity. This may be addressed by testing for endocrine activity in the presence and absence of S9. Research is also required to explore more refined methods that may mimic the *in vivo* situation more precisely than S9. These should include biotechnological approaches, although these could be expensive.

How this might be combined with the current ED tests in validation under the auspices of the OECD can be addressed upon the successful validation of the test methods foreseeable within 2007/2008 (Table 4). As every system behaves differently in different hands, it is important to stress the need for a group of experts to put together a reference chemical set for standardisation, which would include positive and negative controls (at least 25% of the selected chemicals should be negatives), as part of accepted international validation procedures to comparatively assess both the test systems and make interlaboratory comparisons. It is therefore recommended that validation criteria be established prior to undertaking future efforts. That is, a panel of EAS and steroid hormones known to be modulated by metabolism, as well as appropriate non-metabolised EAS and steroid hormones should be established, containing reference potencies and effects of metabolism. Thus research groups attempting to develop such systems can produce comparable data, and a better understanding of the utility of the system should result. The basis of such a panel of test compounds could be the list derived for the ER binding international validation currently being led by the US EPA, and the list for the ER/AR transactivation validation studies being conducted in the EU funded integrated project ReProTect. The testing of panels of positive control compounds should also provide information on the metabolic activity present in the assay system.

Such a chemical panel could also be utilised in the development of *in silico* tools for EA metabolism activity.

The assessment of intrinsic capacity of the cells used in endocrine activity 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 the results obtained. Efforts undertaken in the past to pool and publish the knowledge on metabolic capacity of cell lines used in toxicological research should be continued.

5.2.2. *Medium term goals*

Although the ideal solution for the simultaneous testing of EAS 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. The *in vitro* EAS tests currently being validated internationally under the auspices of the OECD are shown in Table 4. A battery approach would be useful, and is recommended. Criteria that would allow interpretation of the results of combined testing are also required. The possibility to use *in silico* molecular modelling docking studies and (Q)SARs for the analysis of potential EA, 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 for the prediction of metabolism of chemicals by phase I and II enzymes, should continue to be explored and developed. In particular, the metabolic capacity of the assay system could be assessed by comparison of the results obtained *in vitro* with those expected as generated by the *in silico* prescreen. The information generated from the new *in vitro* test could then also be used to refine the *in silico* molecular models and refine and update the QSARs. Thus it might be possible in a relatively 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 for the development of a combined test.

As separate testing for endocrine action 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 EAS *in vitro* tests and from metabolism tests could be used to prioritise potential EAS for further testing. In the medium term, tests should be made available that combine both testing for metabolism and endocrine effects. Studies

should be initiated to obtain greater knowledge about the incorporation of metabolic capacity into existing functional tests, or the incorporation of endocrine molecular targets into existing cell lines with metabolic capacity. Selected referenced examples are presented in Table 6, and examples of typical activity assays and availability of commercially available antibodies for the analyses of xenobiotic metabolising P450s are given in Table 7. These examples give an indication of the more quantitative information that would be useful for later proposals.

In addition, although the use of “mini-arrays” and similar RT-PCR based systems is in its infancy for large scale testing, in the future it should be borne in mind that the same restrictions will apply to tests with these endpoints.

If agreement on a testing framework can be obtained, this might also be helpful to identify those tests that may be of utmost need, and so should be developed with the highest priority. It is suggested that primary *in vitro* tests for endocrine activity at different targets, as described in section 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 such as the ER β , progesterone, glucocorticoid, mineralocorticoid and 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. 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 enzymes. *In vitro* tests to detect metabolism of chemicals could be developed along the lines discussed, but evidently any information on metabolism that is available from other assays could be used at present to decide about the potential EA effects of certain chemicals. *In vitro* tests to detect the interference of chemicals with steroid and thyroid hormone synthesis and metabolism are in development and prevalidation, and the relevant separate DRPs should be consulted, in conjunction with this DRP.

At present most attention is given to estrogenic, androgenic and thyroid activity but other hormonal and ED pathways may be envisaged. If there is activity of concern in *in vitro* tests, according to the OECD Conceptual Framework, the substance should be considered for further testing *in vivo*. Possible tests could include the uterotrophic assay or Hershberger assay, an enhanced OECD 407, OECD 415, OECD 416, and extended one generation (Level 5).

5.2.3. Long term goals

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 enzymes (e.g. P450, SULTs etc) 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 EAS *in vitro*. Whether a substance is an ED or not can currently only be definitely established by careful *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 vitro*, with materials obtained from the animal species and strain that will be used for the *in vivo* test.

It is also recommended that there should be an investigation into the relevance of using the prostaglandin H synthase (PHS) system for EAS. 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)).

Finally, longer term goals will need to be revisited on a regular basis, as the information base develops. It is recommended that this DRP be revised in the light of new developments, and that this recommendation should be added to the workplan for the EDTA and WNT, with a timeline of five years, as well as remaining as a standing agenda item at the VMG-NA.

6. REFERENCES

- Afzelius, L., Zamora, I., Ridderström, M., Andersson, T.B., Karlen, A. & Masimirembwa C.M. (2001). Competitive CYP2C9 inhibitors: enzyme inhibition studies, protein homology modelling, and three-dimensional quantitative structure-activity relationship analysis. *Molecular Pharmacology*, **59**, 909-919.
- Afzelius, L., Raubacher, F., Karlen, A., Jorgensen, F.S., Andersson, T.B., Masimirembwa, C.M. & Zamora, I. (2004). Structural analysis of CYP2C9 and CYP2C5 and an evaluation of commonly used molecular modelling techniques. *Drug Metabolism and Disposition*, **32**, 1218-1229.
- Akingbemi, B.T., Ge, R.-S., Klinefelter, G.R., Gunsalus, G.L. & Hardy, M.P. (2000). A metabolite of methoxychlor, 2,2-Bis(p-hydroxyphenyl)-1,1,1-trichloroethane, reduces testosterone biosynthesis in rat Leydig cells through suppression of steady-state messenger ribonucleic acid levels of the cholesterol side-chain cleavage enzyme. *Biology of Reproduction* **62**, 571-578.
- Altuntas, T. G., Zager, R. A., and Kharasch, E. D. (2003). Cytotoxicity of S-conjugates of the sevoflurane degradation product fluoromethyl-2,2-difluoro-1-(trifluoromethyl) vinyl ether (Compound A) in a human proximal tubular cell line. *Toxicology and Applied Pharmacology* **193**, 55-65.
- Anders, M. W., Lash, L. H., and Elfarra, A. A. (1986). Nephrotoxic amino acid and glutathione S-conjugates: Formation and renal activation. In *Biological Reactive Intermediates III: Mechanism of Action in Animal Models and Human Disease (Series: Advances in Experimental Medicine and Biology)* (J. J. Kocsis, D. J. Jollow, C. M. Witmer, J. O. Nelson, and R. Snyder, Eds.), pp. 443-455.
- Andersen, H. R., Andersson, A.-M., Arnold, S.F., Autrup, H., Barfoed, M., Beresford, N.A., Bjerregaard, P., Christiansen, L.B., Gissel, B., Hummel, R., Jørgensen, E.B., Korsgaard, B., Le Guevel, R., Leffers, H., McLachlan, J., Møller, A., Nielsen, J.B., Olea, N., Oles-Karasko, A., Pakdel, F., Pedersen, K.L., Perez, P., Skakkeboek, N.E., Sonnenschein, C., Soto, A.M., Sumpter, J.P., Thorpe, S.M. & Grandjean, P. (1999). Comparison of short-term estrogenicity tests for identification of hormone-disrupting chemicals. *Environmental Health Perspectives* **107 S1**, 89-108.
- Anon (2001a). US Environmental Protection Agency (2001). *Endocrine disruptor screening program*. www.epa.gov/scipoly/oscp/endo/index.html
- Anon (2001b). Communication for the Commission to the Council and the European Parliament, On the Implementation of the Community Strategy for Endocrine Disruptors: *A range of Substances Suspected of Interfering with the Hormone Systems of Humans and Wildlife (COM(1999)706)*, Commission of the European Communities COM(2001)262 final.
- Assembly of Life Sciences Chapter 4: The Chemistry of nitrate, nitrite, and nitrosylation. The health effects of nitrate, nitrite, and N-nitroso compounds: part 1 of a 2-part study by the committee on nitrite and alternative curing agents in food. *Washington, DC: National Academy Press; 1981; p 4-1 - 4-52*

Assembly of Life Sciences Chapter 6: Environmental distribution and exposure of humans to nitrosatable substrates and modifiers of nitrosation reactions. The health effects of nitrate, nitrite, and N-nitroso compounds: part 1 of a 2-part study by the committee on nitrite and alternative curing agents in food. *Washington, DC: National Academy Press; 1981; p 6-1 - 6-35.*

Ashby, J. (2000). Validation of *in vitro* and *in vivo* methods for assessing endocrine disrupting chemicals. *Toxicological Pathology* **28**, 432-437.

Atkinson, A. & Roy D. (1995). *In vitro* conversion of environmental estrogenic chemical bisphenol A to DNA binding metabolite(s). *Biochemical Biophysical Research Communications* **210**, 424-433.

Atterwill, C. & Flack, J.D. (Eds.) (1992). *Endocrine Toxicology*, Cambridge University Press, p475.

Baker, V. (2001). Endocrine disruptors - testing strategies to assess human hazard. *Toxicology in vitro* **15 (4-5)**, 413-9.

Balaguer, P., Joyeux, A., Denison, M.S., Vincent, R., Gillesby, B.E. & Zacharewski, T. (1996). Assessing the estrogenic and dioxin-like activities of chemicals and complex mixtures using *in vitro* recombinant receptor-reporter gene assays. *Canadian Journal of Physiology and Pharmacology* **74**, 216-222.

Baron, J.M., Höller, D., Schiffer, R., Frankenberg, S., Neis, M., Merk, H.F., and Jugert, F.K. (2001) Expression of multiple Cytochrome P450 Enzymes and Multidrug resistance-associated transport proteins in human skin keratocytes. *J. Invest. Dermatol.* **116**, 541-548.

Belcher, S.M. & Zsarnovszky, A. (2001). Estrogenic actions in the brain: estrogen, phytoestrogens and rapid intracellular signaling mechanisms. *Journal of Pharmacology and Experimental Therapeutics* **299**, 408-414.

Beresford, N., Routledge, E.J., Harris, C.A. & Sumpter J.P. (2000). Issues arising when interpreting results from an *in vitro* assay for estrogenic activity. *Toxicology and Applied Pharmacology* **162**, 22-33.

Bernauer, U., Vieth, B., Ellrich, R., Heinrich-Hirsch, B., Jänig, G.-R. and Gundert-Remy, U. (1999). CYP2E1-dependent benzene toxicity: the role of extrahepatic benzene metabolism. *Arch. Tox.* **73**, 189-196.

Bernauer, U., Vieth, B., Ellrich, R., Heinrich-Hirsch, B., Jänig, G.-R. and Gundert-Remy, U. (2000). CYP2E1 expression in the bone marrow and its intra- and interspecies variability. Approaches for a more reliable extrapolation from one species to another in the risk assessment of chemicals. *Arch Tox.* **73**, 618-624.

Bernauer, U., Ellrich, R., Heinrich-Hirsch, B., Teubner, W., Vieth, B. and Gundert-Remy, U. (2002). Expression of cytochrome P450 enzymes in human colon. *IARC Scientific Publications* **156**, 487-489.

Bernauer, U., Garritsen, H., Heinrich-Hirsch, B. and Gundert-Remy, U. (2003a). Immunochemical analysis of extrahepatic Cytochrome P450 variability in human leucapheresed samples and its consequences for the risk assessment process. *Regulatory Toxicology and Pharmacology* **37**, 318 – 327.

Bernauer, U., Glatt, H-R., Heinrich-Hirsh, B. Liu, Y., Muckel, E., Vieth, B. & Gundert-Remy, U. (2003b). Heterologous expression of mouse cytochrome P450 2E1 in V79 cells: construction and characterisation of the cell line and comparison with V79 cell lines stably expressing rat P450 2E1 and human P450 2E1. *ATLA* **31**, 21-30.

- Blaauboer, B.J., Boobis, A.R., Castell, J.V., Coecke, S., Groothuis, G.M.M., Guillouzo, A., Hall, T.J., Hawksworth, G.M., Lorenzon, G., Miltenburger, H.G., Rogiers, V., Skett, P., Villa, P. & Wiebel, F.J. (1994). The practical applicability of hepatocyte cultures in routine testing. *ATLA* **22**, 231-241.
- Bradlow, H.L., Davis, D.L., Lin, G., Sepkovic, D. & Tiwari, R. (1995). Effects of pesticides on the ratio of 16 α /2-hydroxyestrone: a biologic marker of breast cancer risk. *Environmental Health Perspectives* **103** (S7), 147-150.
- Brandon, E.F., Raap, C.D., Meijerman, I., Beijnen, J.H. & Schellens, J.H. (2003). An update on *in vitro* test methods in human hepatic drug biotransformation research: pros and cons. *Toxicol Appl Pharmacol.* **189**, 233-246
- Brocks, D. R. (2006). Drug disposition in three dimensions: an update on stereoselectivity in pharmacokinetics. *Biopharmaceutics & Drug Disposition*, **27 (8)**: 387-406.
- Brown, S.J., Raja, A.A. & Lewis, D.F.V. (1994). A comparison between COMPACT and Hazard Expert evaluations for 80 chemicals tested by the NTP/NCI rodent bioassay. *ATLA* **22**, 482-500.
- Bulger, W.H. & Kupfer, D. (1990). Studies on the formation of methoxychlor-protein adduct in rat and human liver microsomes. *Biochemical Pharmacology* **40**, 937-945.
- Bulger, W.H., Muccitelli, D. & Kupfer, D. (1978). Interactions of methoxychlor, methoxychlor base-soluble contaminant and 2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane with rat uterine estrogen receptor. *Journal of Toxicology and Environmental Health* **4**, 881-893.
- Bull, S., Langezaal, I., Clothier, R. & Coecke, S. (2001). A genetically engineered cell-based model system for detecting metabolism-mediated toxicity. *ATLA* **29**, 703-716.
- Burchell, B., Brierley, C.H., Monaghan, G. & Clarke, D.J. (1998). The structure and function of the UDP-glucuronosyltransferase gene family. *Advances in Pharmacology* **42**, 335-338.
- Burchell, B., Soars, M., Monaghan, G., Cassidy, A., Smith, D. & Ethell, B. (2000). Drug-mediated toxicity caused by genetic deficiency of UDPglucuronosyltransferases. *Toxicology Letters* **112-113**, 333-340.
- Capen C.C. (1997). Mechanistic data and risk assessment of selected toxic end points of the thyroid gland. *Toxicologic Pathology* **25**, 39-48.
- Cassidy, A. & Milligan, S. (1998). How significant are environmental oestrogens to women? *Climacteric* **1**, 229-242.
- Castell, J. V., Teresa Donato, M., and Gomez-Lechon, M. J. (2005). Metabolism and bioactivation of toxicants in the lung. The *in vitro* cellular approach. *Experimental and Toxicologic Pathology* **57**, 189-204.
- Charles, G.D., Bartels, M.J., Gennings, C., Zacharewski, T.R., Freshour, N.L., Gollapudi, B.B. & Carney, E.W. (2000). Incorporation of S-9 activation into an ER- α transactivation assay. *Reproductive Toxicology* **14**, 207-216.
- Challis B.C. (1973). Rapid nitrosation of phenols and its implications for health hazards from dietary nitrites. *Nature* **244**, 466.

Cheek A.O., Kow K., Chen J. & Mclachlan J.A. (1999). Potential mechanism of thyroid disruption in humans: interaction of organochlorine compounds with thyroid receptor, transthyretin, and thyroid-binding globulin. *Environmental Health Perspectives* **107**, 273-278.

Chen T., Xie W., Agler M., Banks M. (2003) Coactivators in assay design for nuclear hormone receptor drug discovery. *Assay Drug Dev Technol.* **6**:835-42.

Clarke, S.E. (1998). *In vitro* assessment of human cytochrome P450. *Xenobiotica* **28**, 1167-1202.

Coecke, S., Ahr, H., Blaauboer, B.J., Bremer, S., Casati, S., Castell, J., Combes, R., Corvi, R., Crespi, C.L., Cunningham, M.L., Elaut G, Eletti B, Freidig A, Gennari A, Gherzi-Egea JF, Guillouzo A, Hartung T, Hoet P, Ingelman-Sundberg M, Munn S, Janssens W, Ladstetter B, Leahy D, Long A, Meneguz A, Monshouwer M, Morath S, Nagelkerke F, Pelkonen O, Ponti J, Prieto P, Richert L, Sabbioni E, Schaack B, Steiling W, Testai E, Vericat JA, Worth A. (2006) Metabolism: A bottleneck in *in vitro* toxicological test development. The report and recommendations of ECVAM workshop 54. *ATLA* **34**, 1-36.

Coecke, S.; Blaauboer, B.J.; Elaut, G.; Freeman, S.; Freidig, A.; Gensmantel, N.; Hoet, P.; Kapoulas, V.M.; Ladstetter, B.; Langley, G.; Leahy, D.; Mannens, G.; Meneguz, A.; Monshouwer, M.; Nemery, B.; Pelkonen, O.; Pfaller, W.; Prieto, P.; Proctor, N.; Rogiers V.; Rostami-Hodjegan, A.; Sabbioni, E.; Steiling, W. and van de Sandt, J.J.M. (2004) Final Document: Subgroup of Experts on Toxicokinetics and Metabolism in the context of the follow-up of the 7th Amendment on the Cosmetics Directive.

[http://ec.europa.eu/enterprise/cosmetics/doc/antest/\(5\)_chapter_3/9_toxicokinetics_and_metabolism.pdf](http://ec.europa.eu/enterprise/cosmetics/doc/antest/(5)_chapter_3/9_toxicokinetics_and_metabolism.pdf)

Coecke, S., Bogni, A., Worth, A., Hartung, T. & Monshouwer, M. (2001). The use of genetically engineered cells for assessing CYP2D6-related polymorphic effects. *Toxicology in Vitro* **15**, 553-556.

Coecke, S., Catalani, P., Bull, S., Langezaal, I., Bogni, A., Bremer, S. & Balls, M. (2000). The integrated use of a genetically engineered cell line panel harbouring drug enzymes to detect metabolism-related problem compounds. In *Progress in the Reduction, Refinement, and Replacement of Animal Experimentation* (ed. M. Balls, A.-M. van Zeller and M.E. Halder). pp. 147-157. Amsterdam: Elsevier Science.

Coecke, S., Rogiers, V., Bayliss, M, Castell, J., Doehmer, J., Fabre, G., Fry, J., Kern, A. & Westmoreland, C. (1999). The use of long-term hepatocyte cultures for detecting induction of drug enzymes: the current status. ECVAM Hepatocytes and Metabolically Competent Systems Task Force Report 1. *ATLA* **27**, 579-605.

Coldham, N.G., Horton, R., Byford, M.F. & Sauer, M.J. (2002) A binary screening assay for proestrogens in food: metabolic activation using hepatic microsomes and detection with oestrogen sensitive recombinant yeast cells. *Food Add. & Contam.* **19**, 1138-1147.

Combes, R.D. (1992). The *in vivo* relevance of *in vitro* genotoxicity assays incorporating enzyme activation systems. In: G G Gibson (Ed.) *Progress in Drug Metabolism Vol 13*, Taylor and Francis Limited, London, 295 - 321.

Combes, R.D. (2000). Endocrine disruptors: a critical review of *in vitro* and *in vivo* testing strategies for assessing their toxic hazard to humans. *ATLA* **28**, 81-118.

Combes, R. & Balls, M. (2003). How much flexibility is possible when validating new *in vivo* and *in vitro* toxicity test methods? *ATLA* **31**, 225-232.

- Craft E.S., DeVito M.J. & Crofton K.M. (2002). Comparative responsiveness of hypothyroxinemia and hepatic enzyme induction in Long-Evans rats versus C57BL/6J mice exposed to TCDD-like and phenobarbital-like polychlorinated biphenyl congeners. *Toxicological Sciences* **68**, 372-380.
- Cresteil, T. (1998). Onset of xenobiotic metabolism in children: toxicological implications. *Food Add. Contam.* **15**, Supplement, 45-51.
- Cronin, M.T.D. (2001). Computational methods for the prediction of drug toxicity. *Current Opinion in Drug Discovery and Development* **3**, 292-297.
- Cupp, A.S. & Skinner, M.K. (2001). Actions of the endocrine disruptor methoxychlor and its estrogenic metabolite on *in vitro* embryonic rat seminiferous cord formation and perinatal testis growth. *Reproductive Toxicology* **15**, 317-326.
- Davies, E., Tsang, C. -W., Ghazali, A., Harris, R., & Waring, R.H. (2004) Effects of culture with TNF-alpha, TGF-beta and insulin on sulphotransferase (SULT 1A1 and 1A3) activity in human colon and neuronal cell lines. *Toxicol In Vitro.* **18**(6):749-54.
- Dean, B., Chang, S., Doss, G. A., King, C., and Thomas, P. E. (2004). Glucuronidation, oxidative metabolism, and bioactivation of enterolactone in rhesus monkeys. *Archives of Biochemistry and Biophysics* **429**, 244-251.
- Dearden, J.C., Barratt, M.D., Benigni, R., Bristol, D.W., Combes, R.D., Cronin, M.T.D., Judson, P.M., Payne, M.P., Richard, A.M., Tichy, M., Worth, A.P., Yourick, J.J. (1997). The development and validation of expert systems for predicting toxicity. The report and recommendations of an ECVAM/ECB workshop (ECVAM workshop 24). *ATLA* **25**, 223-252.
- Dehal, S.S. & Kupfer, D. (1997). CYP2D6 catalyses tamoxifen 4-hydroxylation in human liver. *Cancer Res.* **57**, 3402-3406.
- Dehal, S.S. & Kupfer, D. (1999). Cytochrome 3A and 2D6 catalyse *ortho*-hydroxylation of 4-hydroxytamoxifen and 3-hydroxytamoxifen (droloxifene): Involvement of catechols in covalent binding to hepatic proteins. *Drug Metabol. Dispos* **27**, 681-688.
- Dekant, W. (2003) Biosynthesis of toxic glutathione conjugates from halogenated alkenes: Cytochrome P450 and Biomonitoring: Mechanisms, Models and Ethical Aspects. *Toxicology Letters* **144**(1), 49-54.
- Demirpence, E., Pons, M., Balaguer, P., & Gagne D. (1992). Study of an antiestrogenic effect of retinoic acid in MCF-7 cells. *Biochem. Biophys. Res. Commun.* **183**, 100-106.
- DeVito M., Biegel L., Brouwer A., Brown S., Brucker-Davis F., Cheek A.O., Christensen R., Colborn T., Cooke P., Crissman J., Crofton K., Doerge D., Gray E., Hauser P., Hurley P., Kohn M., Lazar J., McMaster S., McClain M., McConnell E., Meier C., Miller R., Tietge J. & Tyl R. (1999). Screening methods for thyroid hormone disruptors. *Environmental Health Perspectives* **107**, 407-415.
- Ding, X. and Kaminsky, L.S. (2003). Human extrahepatic cytochromes P450: function in xenobiotic metabolism and tissue-selective chemical toxicity in the respiratory and gastrointestinal tracts. *Annual Review of Pharmacology and Toxicology* **43**, 149-173.
- de Wildt S.N., Kearns G.L., Leeder J.S. & van den Anker J.N. (1999). Cytochrome P450 3A: ontogeny and drug disposition. *Clin. Pharmacokinet.* **37**, 485-505.

Doehmer, J. (1993). V79 Chinese hamster cells genetically engineered for cytochrome P450 and their use in mutagenicity and metabolism studies. *Toxicology* **82**, 105-118.

Doehmer, J., Buters, J.T., Luch, A., Soballa, V., Baird, W.M., Morisson, H., Stegeman, J.J., Townsend, A.J., Greenlee, W.F., Glatt, H.R., Seidel, A., Jacob, J. & Greim, H. (1999). Molecular studies on the toxifying effects by genetically engineered cytochromes P450. *Drug Metabolism Reviews* **31**, 423-435.

Du, L., Hoffman, S.M.G., and Keeney, D.S. (2004): Epidermal CYP2 family cytochromes P450. *Toxicol. Appl. Pharmacol.* **195**, 278-287.

Dudley M.W., Sheeler C.Q., Wang H. & Khan S. (2000). Activation of the human estrogen receptor by the antiestrogens ICI 182,780 and tamoxifen in yeast genetic systems: implications for their mechanism of action. *Proceedings of the National Academy of Sciences* **97**, 3696-3701.

Dutertre, M. & Smith C.L. (2000). Molecular mechanisms of selective estrogen receptor modulator (SERM) action. *Journal of Pharmacology and Experimental Therapeutics* **295**, 431-437.

Eaton, D.L. & Bammler, T.K. (1999). Concise review of the glutathione S-transferases and their significance to toxicology. *Toxicological Sciences* **49**, 156-164.

EDSTAC Final report (1998). Endocrine Disruptor Screening and Testing Advisory Committee Final Report. <http://www.epa.gov/>

Eertmans, F., Dhooge, W., Stuyvaert, S. & Comhaire, F. (2003). Endocrine disruptors: effects on male fertility and screening tools for their assessment. *Toxicol. in vitro* **17**, 515-524.

Eling, T.E., Thompson, D.C., Foureman, G.L., Curtis, J.F. & Hughes, M.F. (1990). Prostaglandin synthase and xenobiotic oxidation. *Ann. Revs. Pharmacol. Toxicol.* **30**, 1-45.

Elliot, B. M., Combes, R. D., Elcombe, C. R., Gatehouse, D. G., Gibson, G. G., Mackay, J. M., and Wolf, R. C. (1992). Alternatives to Arochlor 1254-induced S9 in *in vitro* genotoxicity assays. *Mutagenesis*, **7**, 175-177.

Elmarakby, S. A., Shappell, N. W., Kumar, S., and Sikka, H. C. (1995). Comparative metabolism of 2-acetylaminofluorene by rainbow trout and rat liver subcellular fractions. *Aquatic Toxicol* **33**, 1-15.

Elsby, R., Maggs, J.L., Ashby, J., Paton, D., Sumpter, J.P. & Park, B.K. (2001a). Assessment of the effects of metabolism on the estrogenic activity of xenoestrogens: a two-stage approach coupling human liver microsomes and a yeast estrogenicity assay. *Journal Pharmacology Experimental Therapeutics* **296**, 329-337.

Elsby, R., Maggs, J.L., Ashby, J. & Park, B.K. (2001b). Comparison of the modulatory effects of human and rat liver microsomal metabolism on the estrogenicity of bisphenol A: implications for extrapolation to humans. *Journal Pharmacology Experimental Therapeutics* **297**, 103-113

European Union's Environment and Health Strategy: SCALE, (2003) accessed 6 July 2007. <http://www.euractiv.com/en/environment/environment-health-strategy-scale/article-117480>
http://europa.eu.int/eur-lex/en/com/cnc/2003/com2003_0338en01.pdf

European Union's Environment and Health Strategy Action Plan, (2004) accessed 6 July 2007. <http://ec.europa.eu/environment/health/pdf/com2004416.pdf>

- Fang, H., Tong, W., Perkins, R., Soto, A.M., Prechti, N.V. & Sheehan, D.M. (2000). Quantitative comparisons of *in vitro* assays for estrogenic activities. *Environmental Health Perspectives* **108**, 723-729.
- Flammang, T.J., Yamazoe, Y., Benson, R.W., Roberts, D.W., Potter, D.W., Chu, D.Z.J., Lang, N.P. & Kadlubar, F. (1989). Arachidonic acid-dependent peroxidative activation of carcinogenic arylamines by extrahepatic human tissue microsomes. *Cancer Res.* **49**: 1977-1982.
- Fowles, J.R., Fairbrother, A., Baecher-Steppan, L. & Kerkvliet, N.I. (1994). Immunologic and endocrine effects of the flame-retardant pentabromodiphenyl ether (DE-71) in C57BL/6J mice. *Toxicology* **86**, 49-61.
- Freyberger A. & Scholz G. (2004). Endocrine toxicology-Contributions of *in vitro* methods to the 3R concept. *Altex*, **21**, suppl. Linz 03/2004, 20-27.
- Freyberger, A., & Schmuck, G. (2005). Screening for estrogenicity and anti-estrogenicity: a critical evaluation of an MVLN cell-based transactivation assay. *Toxicology Letters*, **155**, 1-13.
- Friedberg, T., Pritchard, M.P., Bandera, M., Hanlon, S.P., Yao, D., McLaughlin, L.A., Ding, S., Burchell, B. & Wolf, C.R. (1999). Merits and limitations of recombinant models for the study of human P450-mediated drug metabolism and toxicity: an intralaboratory comparison. *Drug Metab Rev* **31**, 523-544.
- Friedman, D., Hu, Z., Kolb, E.A., Gorfajn, B. & Scotto, K.W. (2002). Ecteinascidin-743 inhibits activated but not constitutive transcription. *Cancer Res.* **62**, 3377-3381.
- Fritsche, E., Cline, J.E., Nguyen, N-H., Scanlan, T.S., & Abel, J. (2005). Polychlorinated biphenyls disturb differentiation of normal human neural progenitor cells: clue for involvement of thyroid hormone receptors. *Environmental Health Perspectives* **113**, 871-876.
- Fujimoto, T., Kitamura, S., Sanoh, S., Sugihara, K., Yoshihara, S., Fujimoto, N. & Ohta, S. (2003). Estrogenic activity of an environmental pollutant, 2-nitrofluorene, after metabolic activation by rat liver microsomes. *Biochem Biophys Res Commun.* **303**, 419-426.
- Fukata, H., Miyagawa, H., Yamazaki, N., and Mori, C. 2006. Comparison of ELISA- and LC-MS-based methodologies for the exposure assessment of bisphenol A. *Toxicol. Mech. Meth.* **16**, 427-430.
- Furukawa M., Nishimura M., Ogino D., Chiba R., Ikai I., Ueda N., Naito S., Kuribayashi S., Moustafa M.A., Uchida T., Sawada H., Kamataki T., Funae Y. & Fukumoto M. (2004) Cytochrome P450 gene expression levels in peripheral blood mononuclear cells in comparison with the liver. *Cancer Sci.* **95**, 520-529.
- Gaido, K.W., Leonard, L.S., Maness, S.C., Hall, J.M., McDonnell, D.P., Saville, B & Safe, S. (1999). Differential interaction of the methoxychlor metabolite 2,2-Bis-(p-hydroxyphenyl)-1,1,1-trichloroethane with estrogen receptors α and β . *Endocrinology* **140**, 5746-5753.
- Gaido, K.W., Maness, S.C., McDonnell, D.P., Dehal, S.S., Kupfer, D & Safe, S. (2000). Interaction of methoxychlor and related compounds with estrogen receptor α and β , and androgen receptor: structure-activity studies. *Molecular Pharmacology* **58**, 852-858.
- Gebhardt, R., Lippert, C., Schneider, A. & Doehmer, J. (1999). Improved determination of drug metabolism by perfusion of recombinant V79 cells carrying human CYP3A4. *Toxicol. in vitro* **13**, 639-643.

- Gebhardt, R., Hengstler JG, Müller D, Glöckner R, Buenning P, Laube B, Schmelzer E, Martina Ullrich, Utesch D, Hewitt N, Ringel M, Reder Hilz B, Bader A, Langsch A, Koose T, Burger HJ, Maas J, Oesch F, (2003) New hepatocyte *in vitro* systems for drug metabolism: Metabolic capacity and recommendations for application in basic research and drug development, standard operation procedures, *Drug Metabolism Reviews*, **35**, 145-213.
- Ghaoui, R., Sallustio, B. C., Burcham, P. C., and Fontaine, F. R. (2003). UDP-glucuronosyltransferase-dependent bioactivation of clofibrac acid to a DNA-damaging intermediate in mouse hepatocytes. *Chemico-Biological Interactions* **145**, 201-211.
- Glatt H. (2000). Sulfotransferases in the bioactivation of xenobiotics. *Chemico- Biological Interactions* **129**, 141-70.
- Glatt, H., Boeing, H., Engelke, C. E. H., Ma, L., Kuhlow, A., Pabel, U., Pomplun, D., Teubner, W., and Meinl, W. (2001). Human cytosolic sulphotransferases: genetics, characteristics, toxicological aspects: Genetic Susceptibility to Environmental Toxicants. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* **482**, 27-40.
- Glatt, H., Schneider, H. and Liu, Y (2005) V79-hCYP2E1-hSULT1A1, a cell line for the sensitive detection of genotoxic effects induced by carbohydrate pyrolysis products and other food-borne chemicals. *Mutation Research* **580**, 41 – 52.
- Graham, J.D. & Clarke, C.L. (1997). Physiological action of progesterone in target tissues. *Endocr Rev.* **18**, 502-519.
- Gray, L.E. Jr., Kelce, W.R., Wiese, T., Tyl, R., Gaido, K., Cook, J., Klinefelter, G., Desaulniers, D., Wilson, E., Zacharewski, T., Waller, D., Foster, P., Laskey, J., Reel, J., Giesy, J., Laws, S., McLachlan, J., Breslin, W., Cooper, R., Di Giulio, R., Johnson, R., Purdy, R., Mihaich, E., Safe, S., Sonnenschein, C., Welshons, W., Miller, R., McMaster, S. & Colborn, T. (1997). Endocrine screening methods Workshop Report: detection of estrogenic and androgenic hormonal and antihormonal activity for chemicals that act via receptor or steroidenic enzyme mechanisms. *Reproductive Toxicology* **11**, 719-750.
- Gomez-Lechon, M.J., Donato, T., Jover, R., Rodriguez, C., Ponsoda, X., Glaise, D., Castell, J.V. & Guguen-Guillouzo, C. (2001). Expression and induction of a large set of drug- enzymes by the highly differentiated human hepatoma cell line BC2. *European Journal of Biochemistry* **268**, 1448-1459.
- Goodman and Gilman's, *The Pharmacological Basis of Therapeutics*, 11th edition, Laurence L. Brunton, PhD (ed), McGraw-Hill, NY 2006
- Goodwin, B., Hodgson, E., D'Costa, D.J., Robertson, G.R., Liddle, C. (2002). Transcriptional regulation of the human CYP3A4 gene by the constitutive androstane receptor. *Mol. Pharmacol.* **62**, 359-365.
- Greene, N., Judson, P.N., Langowski, J.J. & Marchant, C.A. (1999). Knowledge-based expert systems for toxicity and metabolism predictions: DEREK, StAR and METEOR. *SAR & QSAR Environmental Research* **10**, 299-314.
- Guengerich, F.P. (2005). Principles of covalent binding of reactive metabolites and examples of activation of bis-electrophiles by conjugation. *Arch. Biochem. Biophys.* **15**, 369-378.
- Hägglad, J., Carlsson, B., Kivelä, P. & Siitari, H. (1995). Novel non-separation binding assays with scintillation microtitration plates: determination of equilibrium and kinetic hormone binding constants for recombinantly produced human estrogen receptor hormone binding domain. *Biotechniques* **18**, 146-151.

- Hakkola, J., Pelkonen, O., Pasanen, M. & Raunio, H. (1998). Xenobiotic- cytochrome P450 enzymes in the human feto-placental unit: role in intrauterine toxicity. *Crit. Rev. Toxicol.* **28**, 35-72.
- Hahn, M.E. (1998). The aryl hydrocarbon receptor: a comparative perspective. *Comparative biochemistry and physiology Part C* **121**, 23-53.
- Hahn, M.E. (2002). Aryl hydrocarbon receptors: diversity and evolution. *Chemico-Biological Interactions* **141**, 131-160.
- Handschin, C. & Meyer, U.A. (2003). Induction of drug metabolism: the role of nuclear receptors. *Pharmacological Reviews* **55**, 649-673.
- Harrington, W.R., Kim, S.H., Funk, C.C., Madak-Erdogan, Z., Schiff, R., Katzenellenbogen, J.A., Katzenellenbogen, B.S. (2006) Estrogen dendrimer conjugates that preferentially activate extranuclear, nongenomic versus genomic pathways of estrogen action. *Mol Endocrinol.* **20**(3):491-502.
- Harris, C.A., Henttu, P., Parker, M.G. & Sumpter, J.P. (1997). The estrogenic activity of phthalate esters *in vitro*. *Environmental Health Perspectives* **105**, 802-811.
- Harris, R.M., Kirk, C.J., and Waring, R.H. (2005) Non-genomic effects of endocrine disruptors: inhibition of estrogen sulfotransferase by phenols and chlorinated phenols. *Mol Cell Endocrinol.* **1;244(1-2)**, 2-4.
- Hashimoto, Y., Moriguchi, Y., Oshima, H., Kawaguchi, M., Miyazaki, K. & Nakamura, M. (2001). Measurement of estrogenic activity of chemicals for the development of new dental polymers. *Toxicology In Vitro* **15**, 421-425.
- Hasenbrink, G., Sievernich, A., Wildt, L., Ludwig, J., Lichtenberg-Frate, H. (2006) Estrogenic effects of natural and synthetic compounds including tibolone assessed in *Saccharomyces cerevisiae* expressing the human estrogen alpha and beta receptors. *FASEB J.* **20**(9), 1552-4.
- Hatagima, A. (2002) Genetic polymorphisms and metabolism of endocrine disruptors in cancer susceptibility. *Cad Saude Publica.* **18**(2), 357-77.
- Hasbi A., O'Dowd B.F. & George S.R. (2005). A G protein-coupled receptor for estrogen: the end of the search. *Mol. Interventions* **5**, 158-161.
- Hawkins M.B., Thornton J.W., Crews D., Skipper J.K., Dotte A. & Thomas P. (2000). Identification of a third distinct estrogen receptor and reclassification of estrogen receptors in teleosts. *Proceedings of the National Academy of Sciences* **97**, 10751-10756.
- Hawkins M.B. & Thomas P. (2004). The unusual binding properties of the third distinct teleost estrogen receptor subtype ERbeta are accompanied by high conserved amino acid changes in the ligand binding domain. *Endocrinology* **145**, 2968-2977.
- Hellmold, H., Lamb, J.G., Wyss, A., Gustafsson, J.A. & Warner, M. (1995). Developmental and endocrine regulation of P450 isoforms in rat breast. *Mol. Pharmacol.* **48**, 630-638.

Hellmold, H., Magnusson, M., Pelto-Huikko, M., Rylander, T., Gustafsson J.A. & Warner M. (1998a). Identification of CYP2A3 as a major cytochrome P450 enzyme in the female peripubertal rat breast. *Mol. Pharmacol.* **53**, 475-482.

Hellmold, H., Rylander, T., Magnusson, M., Reihner, E., Warner M. & Gustafsson J.A. (1998b). Characterisation of cytochrome P450 enzymes in human brEASt tissue from reduction mammoplasties. *J. Clin. Endocrin. Metabolism*, **83**, 886-895.

Hengstler, J.G., Utesch, D., Steinberg, P., Platt, K.L., Diener, B., Ringel, M., Swales, N., Fischer, T., Biefang, K., Gerl, M., Bottger, T. & Oesch, F. (2000). Cryopreserved primary hepatocytes as a constantly available *in vitro* model for the evaluation of human and animal drug metabolism and enzyme induction. *Drug Metabolism Reviews* **32**, 81-118.

Hengstler, J.G, Brulport M, Schormann W, Bauer A, Hermes M, Nussler AK, Fandrich F, Ruhnke M, Ungefroren H, Griffin L, Bockamp E, Oesch F, von Mach M.-A. (2005). Generation of human hepatocytes by stem cell technology: definition of the hepatocyte. *Expert Opin. Drug Metab. Toxicol.* **1**, 61-74.

Hewitt, N.J., Lechon, M.J., Houston, J.B., Hallifax D, Brown HS, Maurel P, Kenna JG, Gustavsson L, Lohmann C, Skonberg C, Guillouzo A, Tuschl G, Li AP, LeCluyse E, Groothuis GM, Hengstler JG. (2007) Primary hepatocytes: current understanding of the regulation of metabolic enzymes and transporter proteins, and pharmaceutical practice for the use of hepatocytes in metabolism, enzyme induction, transporter, clearance, and hepatotoxicity studies. *Drug Metab Rev.* **39(1)**, 159-234.

Hines, R.N., Cresteil, L.T., Ding, X., Prough, R.A., Fitzpatrick, J.L., Ripp, S.L., Falkner, C., Ge, N.-L., Levine, A. & Elferink, C.J. (2001). Molecular regulation of genes encoding xenobiotic- enzymes: mechanisms involving endogenous factors. *Drug Metabolism and Disposition* **29**, 623-633.

Hines, R.N. & McGarver D.G. (2002). The ontogeny of human drug- enzymes: phase I oxidative enzymes. *Journal Pharmacology Experimental Therapeutics* **300**, 355-360.

Honkakoski, P. & Negishi, M. (2000). Regulation of cytochrome P450 (CYP) genes by nuclear receptors. *Biochem. J.* **347**, 321-337.

Hornhardt, S. & Wiebel, F.J. (1996). CCLTOP Catalogue of Cell Lines in Toxicology & Pharmacology, First Edition, ISSN 0721-1694. GSF-Bericht 3/96 GSF-Forschungszentrum für Umwelt und Gesundheit. Germany pp249.

Hotchkiss, S.A.M. (1992). Skin as a xenobiotic organ. In: G G Gibson (Ed.) *Progress in Drug Metabolism Vol 13*, Taylor and Francis Limited, London, 217-262.

Houston, J.B. & Carlile D.J. (1997). Prediction of hepatic clearance from microsomes, hepatocytes and liver slices. *Drug Metab. Rev.* **29**, 891-922.

Howdeshell, K.L. (2002). A model of the development of the brain as a construct of the thyroid system. *Environmental Health Perspectives* **110, Suppl.3**, 337-348

Hu, Y. & Kupfer, D. (2002a). Metabolism of the endocrine disruptor pesticide-methoxychlor by human P450S: pathways involving a novel catechol metabolite. *Drug Metabolism and Disposition* **30**: 1035-1042.

Hu, Y. & Kupfer, D. (2002b). Enantioselective metabolism of the endocrine disruptor pesticide methoxychlor by human cytochromes P450 (P450S): major differences in selective enantiomer formation by various P450 isoforms. *Drug Metabolism and Disposition* **30**: 1329-1336.

Iannone, M.A., Consler, T.G., Pearce, K.H., Stimmel, J.B., Parks, D.J., Gray, J.G. (2001) Multiplexed molecular interactions of nuclear receptors using fluorescent microspheres. *Cytometry*. **44**:326-37.

ICCVAM (2003). ICCVAM evaluation of *in vitro* test methods for detecting potential endocrine disruptors: estrogen receptor and androgen receptor binding and transcriptional activation assays. NIH Publication No: 03-4503.

Irving, C. C. (1971). Metabolic activation of N-hydroxy compounds by conjugation. *Xenobiotica* **1**, 387-398.

Ito, K., Iwatsubo, T., Kanamitsu, S., Nakajima, Y. & Sugiyama, Y. (1998). Quantitative prediction of *in vivo* drug clearance and drug interactions from *in vitro* data on metabolism, together with binding and transport. *Annu. Rev. Pharmacol. Toxicol.* **38**, 461-499.

Jacobs M.N. & Lewis, D.V.L. (2002) Steroid hormone receptors and dietary ligands: a selected review. *Proc. Nut. Soc.* **61**, 105-122

Jacobs, M.N., Dickins, M., & Lewis, D.F.V. (2003) Homology modelling of the nuclear receptors: human oestrogen receptor β (hER β), the human pregnane-x-receptor (PXR), The Ah receptor (AhR) and the constitutive androstane receptor (CAR) ligand binding domains from the human oestrogen receptor α (hER α) crystal structure, and the human peroxisome proliferator activated receptor α (PPAR α) ligand binding domain from the human PPAR γ crystal structure. *Journal of Steroid Biochemistry and Molecular Biology*. **84/2**, 117-132.

Jacobs, M.N. (2004) In silico tools to aid risk assessment of endocrine disrupting chemicals. *Toxicology*. **205(1-2)**, 43-53.

Jacobs, M.N. (2005) Nuclear receptors and dietary ligands. In 'Nutrients and Cell Signaling', ed: J Zemplini and K Dakshinamurti, 'Oxidative Stress and Disease' series. pp 35-90. publ. CRC Press, Taylor and Francis Limited, Boca Raton, FL.

Jacobs, M.N., Nolan, G.T., & Hood, S.R. (2005). Lignans, bacteriocides and organochlorine compounds activate the human Pregnane X Receptor. *Toxicol. App. Pharmacol.* **1;209(2)**, 123-33

Janjua, N. R., Krogh Mortensen, G., Andersson, A-M., Kongshoj, B., Skakkebæk, N.E., Wulf, H.E. (2007) Systemic uptake of diethyl phthalate, dibutyl phthalate, and butyl paraben following whole-body topical application and reproductive and thyroid hormone levels in humans. *Environmental Science and Technology*, **41(15)**, 5564-70.

Jaworska, J.S., Dimitrov, S., Nikolova, N., Mekenyan, O. (2002). Probabilistic assessment of biodegradability based on metabolic pathways: CATABOL system. *SAR QSAR Environ Res.* **13(2)**, 307-23.

Jenkins, J.S. & Hall, C.J. (1977) Metabolism of [14 C] testosterone by human foetal and adult brain tissue. *J. Endocrinol.* **74**, 425-429.

Johansson, M., Nilson, S. & Lund, B.-O. (1998). Interactions between methylsulfonyl PCBs and the glucocorticoid receptor. *Environmental Health Perspectives* **106**, 769-772.

Johansson, M.K., Sanderson, J.T. & Lund, B-O. (2002). Effects of 3-MeSO₂-DDE and some CYP inhibitors on glucocorticoid steroidogenesis in the H295R human adrenocortical carcinoma cell line. *Toxicology In vitro* **16**, 113-121.

Jordan, V.C. & Morrow, M. (1999). Tamoxifen, raloxifene, and the prevention of breast cancer. *Endocrine Reviews*, **20**, 253-278.

Joyeux, A., Balauer, P., Germain, P., Boussioux, A.M., Pons, M. & Nicholas, J.C. (1997). Engineered cell lines as a tool for monitoring biological activity of hormone analogs. *Analytical Biochemistry* **249**, 119-130.

Julien, E., Willhite, C.C., Richard, A.M., Desesso, J.M. (2004) Challenges in constructing statistically based structure-activity relationship models for developmental toxicity. *Birth Defects Res A Clin Mol Teratol.*; **70(12)**:902-11.

Kamp, H., Heuner, H-A., Verlohner, A., Leibold, A., and van Ravenzwaay, B (2005). In house validation of a yeast-based assay to determine (anti)estrogenic and (anti) androgenic potential. *ALTEX* **22**, special issue 122.

Kassahun, K., Pearson, P.G., Tang, W., McIntosh, I., Leung, K., Elmore, C., Dean, D., Wang, R., Doss, G., Baillie, T.A. (2001) Studies on the metabolism of troglitazone to reactive intermediates *in vitro* and *in vivo*. Evidence for novel biotransformation pathways involving quinone methide formation and thiazolidinedione ring scission. *Chem Res Toxicol.* **14(1)**:62-70.

Kato, Y., Haraguchi, K., Shibahara, T., Yumoto, S., Masuda, Y. & Kimura, R. (2000). Reduction of serum thyroxine concentrations by methylsulfonyl metabolites of tetra-, penta- and hexachlorinated biphenyls in male Sprague-Dawley rats. *Chemosphere* **40**, 1233-1240.

Kauffman, R.F., Bensch, W.R., Roudebush, R.E., Cole, H.W., Bean, J.S., Phillips, D.L., Monroe, A., Cullinan, G.J., Glasebrook, A.L. & Bryant, H.U. (1997). Hypocholesteremic activity of raloxifene (LY139481): pharmacological characterisation as a selective estrogen receptor modulator. *Journal of Pharmacology and Experimental Therapeutics* **280**: 146-153.

Kester, M.H.A., Bulduk, S., Tibboel, D., Meinel, W., Glatt, H., Falany, C.N., Coughtrie, M.W.H., Bergman, A., Safe, S.H., Kuiper, G.G.J.M., Schuur, A.G., Brouwer, A. & Visser, T.J. (2000). Potent inhibition of estrogen sulfotransferase by hydroxylated PCB metabolites: a novel pathway explaining the estrogenic activity of PCBs. *Endocrinology*, **141**, 1897-1900.

Kester, M.H.A., Bulduk, S., Van Toor, H., Tibboel, D., Meinel, W., Glatt, H., Falany, C.N., Coughtrie, M.W.H., Schuur, A.G., Brouwer, A. & Visser, T.J. (2002). Potent inhibition of estrogen sulfotransferase by hydroxylated metabolites of polyhalogenated aromatic hydrocarbons reveals alternative mechanism for estrogenic activity of endocrine disrupters. *J. Clin. Endocrinol. & Metabol.* **87**, 1142-1150.

King, C. M., and Glowinski, I. B. (1983). Acetylation, deacetylation and acyltransfer. *Environmental Health Perspectives* **49**, 43-50.

Kirk, C.J., Bottomley, L., Minican, N., Carpenter, H., Shaw, S., Kohli, N., Winter, M., Taylor, E.W., Waring, R.H., Michelangeli, F., Harris, R.M. (2003) Environmental endocrine disrupters dysregulate estrogen metabolism and Ca²⁺ homeostasis in fish and mammals via receptor-independent mechanisms. *Comp Biochem Physiol A Mol Integr Physiol.* **135(1)**:1-8.

- Kitamura, S., Ohmegi, M., Sanoh, S., Sugihara, K., Yoshihara, S., Fujimoto, N. & Ohta, S. (2003). Estrogenic activity of styrene oligomers after metabolic activation by rat liver microsomes. *Environmental Health Perspectives* **111**, 329-334.
- Klopman, G., Diumayuga, M. & Galafgous, J. (1994). META. 1. A program for the evaluation of metabolic transformation of chemicals. *Journal of Chemical Information and Computer Sciences* **34**, 1320-1325.
- Kohno, H., Gandini, O., Curtis, S.W. & Korach, K.S. (1994). Antiestrogen activity in the yeast transcription system: estrogen receptor mediated agonist response. *Steroids* **59**, 572-578.
- Kronbach, T., Larabee, T.M. & Johnson, E.F. (1989). Hybrid cytochromes p-450 identify a substrate binding domain in P-450Ic5 and 450IIC4. *Proc. Natl. Acad. Sci. USA* **86**, 8262-8265.
- Kuil, C.W., Brouwer, A., van der Saag, P.T., van der Burg, B. (1998). Interference between progesterone and dioxin signal transduction pathways. *J. Biol. Chem.* **273**, 8829-8834.
- Kuiper G.G.J.M., Lemmen J.G., Carlson B., Corton J.C., Safe S.H., Van der Saag P.T., Van der Burg B. & Gustafsson J.-A. (1998). Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β . *Endocrinology* **139**, 4252-4263.
- Kupfer, D. & Bulger, W.H. (1987). Metabolic activation of pesticides with prestrogenic activity. *Fed. Proc.* **46**: 1864-1869.
- Kupfer, D. & Dehal, S.S. (1996). Tamoxifen metabolism by microsomal cytochrome P450 and FMO. "Methods in Enzymology" (Cytochrome P450, Pt. B; E.F. Johnson & M.R. Waterman, Eds.) **272**, 152-163.
- Kurebayashi, H., Betsui, H. and Ohno, Y. (2003). Disposition of a low dose of ^{14}C -bisphenol A in male rats and its main biliary excretion as BPA-glucuronide. *Toxicol. Sci.* **73**,17-25.
- Lai, K.P., Wong, M.H. & Wong, C.K.C. (2004). Modulation of AhR-mediated CYP1A1 mRNA and EROD activities by 17β -estradiol and dexamethasone in TCDD-induced H411E cells. *Tox. Sciences* **78**, 41-49.
- Lampe, J.W., King, I., Li, S., Grate, M.T., Barale, K.V., Chen, C., Feng, Z. & Potter, J.D. (2000). Brassica vegetables increase and apiaceous vegetables decrease cytochrome P450 1A2 activity in humans: changes in caffeine metabolite ratios in response to controlled vegetable diets. *Carcinogenesis* **21**, 1157-1162.
- Lanza, D.L., Code, E., Crespi, C.L., Gonzalez, F.J. & Yost, G.S. (2004). Specific dehydrogenation of 3-methylindole and epoxidation of naphthalese by recombinant human CYP2F1 expressed in lymphoblastoid cells. *Drug Metab Disp* **27**, 798-803.
- Lazar M.A. (1993). Thyroid hormone receptors: multiple forms, multiple possibilities. *Endocrine Reviews* **14**, 184-193.
- LeCluyse, E.L. (2001). Human hepatocyte culture systems for the *in vitro* evaluation of cytochrome P450 expression and regulation. *European Journal of Pharmaceutical Sciences* **13**, 343-368.
- Legler, J., Van den Brink, C.E., Brouwer, A., Murk, A.J., Van der Saag, P.T., Vethaak, A.D. & Van der Burg, B. (1999). Development of a stably transfected estrogen receptor-mediated luciferase reporter gene assay in the human T47-D breast cancer cell line. *Toxicological Sciences*, **48**, 55-66.

Legler, J., Dennekamp, M., Vethaak, A.D., Brouwer, A., Koeman, J.H., Van der Burg, B., Murk, A.J. (2002a). Detection of estrogenic activity in sediment-associated compounds using *in vitro* reporter gene assays. *Sci. Total Environ.* **293**, 69-83.

Legler, J., Zeinstra, L.M., Schuitemaker, F., Lanser, P.H., Bogerd, J., Brouwer, A., Vethaak, A.D., De Voogt, P., Murk, A.J. & Van der Burg, B. (2002b). Comparison of *in vivo* and *in vitro* reporter gene assays for short-term screening of estrogenic activity. *Environmental Science Technology*, **36**, 4410-4415.

Lehmann, J McKee D, Watson, M., Willson, T., Moore, J., Kliewer, SA (1998). The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions. *J Clin Invest* **102**: 1016-1023

Lemmen, J.G., Van den Brink, C.E., Legler, J., Van der Saag, P.T. & Van der Burg, B. (2002). Detection of estrogenic activity of steroids present during mammalian gestation using oestrogen receptor α - and oestrogen receptor β -specific *in vitro* assays. *Journal of Endocrinology* **174**, 435-446.

Letcher, R.J., Lemmen, J.G., Van der Burg, B., Brouwer, A., Bergman, A., Giesy J.P. & Van Den Berg M. (2002). *In vitro* antiestrogenic effects of Aryl Methyl Sulfone metabolites of Polychlorinated Biphenyls and 2,2-Bis(-chlorophenyl)-1,1-dichloroethene on 17 β -estradiol-induced gene expression in several bioassay systems. *Toxicological Sciences*, **69**, 362-372.

Lewis, D.F.V. (1996). *Cytochromes P450, Structure, function and mechanism*. Taylor & Francis, London, 348p.

Lewis, D.F.V., Ioannides, C. & Parke, D.V. (1998). An improved and updated version of the compact procedure for the evaluation of P450-mediated chemical activation. *Drug Metabolism Reviews* **30**, 709-737

Lewis, D.F.V., Lake, B.G., George, S.G., Dickins, M., Eddershaw, P.J., Tarbit, M.H., Beresford, A.P., Goldfarb, P.S. & Guengerich, F.P. (1999). Molecular modelling of CYP1 family enzymes CYP1A1, CYP1A2, CYP1A6, and CYP1B1 based on sequence homology with CYP1O2. *Toxicology* **139**, 53-79.

Lewis, D.F.V. (2003) 'Pesticides and P450 induction: Gender implications' in Jacobs, M.N. and Dinham, B. Eds. Silent Invaders, Pesticides, Livelihoods and Women's Health. 342pp. Publ. Zed books Ltd. London.

Lewis, J.S., Thomas, T.J., Klinge, C.M., Gallo, M.A. & Thomas, T. (2001). Regulation of cell cycle and cyclins by 16 α -hydroxyestrone in MCF-7 breast cancer cells. *J. Mol. Endocrinol.* **27**: 293-307.

Lewis J.S., Thomas T.J., Pestell R.G., Albanese C., Gallo M.A. & Thomas T. (2005). Differential effects of 16 α -hydroxyestrone and 2-methoxyestradiol on cyclin D1 involving the transcription factor ATF-2 in MCF-7 breast cancer cells. *J. Mol. Endocrinol.* **34**: 91-105.

Li, A.P., Lu C., Brent J.A., Pham C., Fackett A., Ruegg C.E. & Silber P.M. (1999). Cryopreserved human hepatocytes: characterisation of drug-metabolising enzyme activities and applications in higher throughput screening assays for hepatotoxicity, metabolic stability, and drug-drug interaction potential. *Chemico-Biological Interactions* **121**, 17-35.

Li, H.-C., Mani, C. & Kupfer D. (1993). Reversible and time-dependent inhibition of the hepatic cytochrome P450 steroidal hydroxylases by the preestrogenic pesticide methoxychlor in rat and human. *J. Biochem. Toxicology* **8**, 195-206.

- Li, X., Bertics, P.J., & Karavolas, H.J. (1997). Regional distribution of cytosolic and particulate 5 α -dihydroprogesterone 3 α -hydroxysteroid oxidoreductases in female rat brain. *J. Steroid Biochem. Mol. Biol.* **60**, 311-318.
- Lin, J.H. & Lu, A.Y.H. (2001). Interindividual variability in inhibition and induction of cytochrome P450 enzymes. *Annu. Rev. Pharmacol. Toxicol.* **41**, 535-567.
- Linget, J.M. & du Vignaud, P. (1999). Automation of metabolic stability studies in microsomes, cytosol and plasma using a 215 Gilson liquid handler. *Journal of Pharmaceutical and Biomedical Analysis* **19**, 893-901.
- Liu, J., Antaya, M., Goff, A.K., Boerboom, D., Silversides, D.W., Lussier, J.G. & Sirois, J. (2001). Molecular Characterisation of Bovine Prostaglandin G/H Synthase-2 and Regulation in Uterine Stromal Cells. *Biology of Reproduction* **64**, 983-991.
- Liu J., Knappenberger K.S., Käck H., Andersson G., Nilsson E., Dartsch C. & Scott C.W. (2003). A homogeneous *in vitro* functional assay for estrogen receptors: coactivator recruitment. *Mol. Endocrinology* **17** (3): 346-355.
- Lopez-Garcia, M. P., Dansette, P. M., and Coloma, J. (2005). Kinetics of tienilic acid bioactivation and functional generation of drug-protein adducts in intact rat hepatocytes. *Biochemical Pharmacology* **70**, 1870-1882.
- Lord, R.S., Bongiovanni, B. & Bralley, J.A. (2002). Estrogen metabolism and the diet-cancer connection: rationale for assessing the ratio of urinary hydroxylated estrogen metabolites. *Alternative Medicine Review* **7**, 112-129.
- Macé, K., Offord, E.A. & Pfeifer, A.M.A. (1997). Drug metabolism and carcinogen activation studies with human genetically engineered cells. In: J.V. Castell & M.J. Gómez-Lechón (Eds.), *In vitro Methods in Pharmaceutical Research*, Academic Press, London, pp. 433-456.
- MacGregor, J.T., Collins, J.M., Sugiyama, Y., Tyson, C.A., Dean, J., Smith, L., Andersen, M., Curren, R.D., Houston, J.B., Kadlubar, F.F., Kedderis, G.L., Krishnan, D., Li, A.P., Parchment, R.E., Thummel, K., Tomaszewski, J.E., Ulrich, R., Vickers, A.E.M. & Wrighton, S.A. (2001). *In vitro* human tissue models in risk assessment: report of a consensus-building workshop. *Toxicological Sciences* **59**, 17-36.
- Mak, P., Dela Cruz, F. & Chen, S. (1999). A yeast screen system for aromatase inhibitors and ligands for androgen receptor: yeast cells transformed with aromatase and androgen receptor. *Environmental. Health. Perspectives.* **107**, 855-860.
- Maness, S.C., McDonell, D.P. & Gaido, K.W. (1998). Inhibition of androgen receptor-dependent transcriptional activity by DDT isomers and methoxychlor in HegG2 human hepatoma cells. *Toxicol. Appl. Pharmacol.* **151**, 135-142.
- Masuda, S., Terashima, Y., Sano, A., Kuruto, R., Sugiyama, Y., Shimo, K., Tanji, K., Yoshioka, H., Terao, Y., & Kinae, N. (2005). Changes in the mutagenic and estrogenic activities of bisphenol A upon treatment with nitrite. *Mutation Res.* **585**:137-146.
- Maslankiewicz, L., Hulzebos, E.M., Vermeire, T.G., Muller, J.J.A., Piersma, A.H., Can chemical structure predict reproductive toxicity. (2005) RIVM rapport 601200005, pp. 76. <http://www.rivm.nl/bibliotheek/rapporten/601200005.html>

Masuyama, H., Hiramatsu, Y., Kunitomi, M., Kudo, T. & MacDonald, P.N. (2000). Endocrine disrupting chemicals, phthalic acid and nonylphenol, activate Pregnane X receptor-mediated transcription. *Molecular Endocrinology* **14**, 421-428.

Matthews, J. & Gustafsson, J.A. (2003). Estrogen signaling: a subtle balance between ER alpha and ER beta. *Mol. Interv.* **3**, 281-292.

Matthews, J., Wihlen, B., Tujague, M., Wan, J., Strom, A., Gustafsson, J-A. (2006). Estrogen Receptor (ER) beta Modulates ERalpha-Mediated Transcriptional Activation by Altering the Recruitment of c-Fos and c-Jun to Estrogen-Responsive Promoters. *Mol Endocrinol.* **20**(3):534-43.

McEwen, B., & Alves, S.E. (1999). Estrogen actions in the central nervous system. *Endocrine Rev.* **20**, 279-307.

McGarver, D.G. & Hines, R.N. (2002). The ontogeny of human drug-etalabolising enzymes; phase II conjugation enzymes and regulatory mechanisms. *Journal Pharmacology Experimental Therapeutics* **300**, 361-366.

McLachlan J.A. (2001). Environmental signaling: What embryos and evolution teach us about endocrine disrupting chemicals. *Endocrine Reviews*, **22**, 319-341.

Meerman, J.H.N., van Lipzig, M.M.H., ter Laak, A.M., Wamelink, M., Geerke, D., Vermeulen, N.P.E. (2003). Formation of estrogenic metabolites from polycyclic aromatic hydrocarbons and halogenated biphenyls by cytochrome P450 activity and development of a predictive computational model for binding to the estrogen receptor. *Toxicology Letters* **144**, S181.

Meerts, I.A.T.M., Letcher, R.J., Hoving, S., Marsh, G., Bergman, A., Lemmen, J.G., Van der Burg, B., & Brouwer, A. (2001). *In vitro* estrogenicity of Polybrominated Diphenyl Ethers, hydroxylated PBDE's, and Polybrominated Bisphenol A compounds. *Environmental Health Perspectives* **109**, 399-407.

Mekenyan, O. G., Dimitrov, S.D., Pavlov, T. S. and Veith, G. D. (2004a). A systems approach to simulating metabolism in computational toxicology. I. The TIMES Heuristic Modelling Framework. *Current Pharmaceutical Design* **10** (11), 1273-1293.

Mekenyan, O.G., Dimitrov, S., Serafimova, R., Thompson, E., Kotov, S., Dimitrova, N., Walker, J. (2004b). Identification of the structural requirements for mutagenicity, by incorporating molecular flexibility and metabolic activation of chemicals I. TA100 Model. *Chem. Res.Toxicol.* **17**(6):753-766 (2004).

Mensah-Nyagan, A.G., Do-Rego, J.-L., Beaujean, D., Luu-The, V., Pelletier, G. & Vaudry, H. (1999). Neurosteroids: Expression of steroidogenic enzymes and regulation of steroid biosynthesis in the central nervous system. *Pharmacological reviews* **51**, 63-81.

Miyazaki, W., Iwasaki, T., Takeshita, A., Kuroda, Y., & Koibuchi, N. (2004). Polychlorinated biphenyls suppress thyroid hormone receptor-mediated transcription through a novel mechanism. *Journal Biological Chemistry* **279**, 18195-18202.

Michnovicz, J.J, Adlercreutz, H., & Bradlow, H.L. (1997). Changes in levels of urinary estrogen metabolites after oral indole-3-carbinol treatment in humans. *J. Natl. Cancer Institute* **89**, 718-723.

- Miller, E. C., Lotlikar, P. D., Miller, J. A., Butler, B. W., Irving, C. C., and Hill, J. T. (1968). Reactions (in vitro) of some tissue nucleophiles with the glucuronide of the carcinogen N-hydroxy-2-acetylaminofluorene. *Mol. Pharmacol.* **4**, 147-154.
- Miller, S., Kennedy, D., Thomson, J., Han, F., Smith, R., Ing, N., Piedrahita, J. & Busbee, D. (2000). A rapid and sensitive reporter gene that uses green fluorescent protein expression to detect chemicals with estrogenic activity. *Toxicological Sciences* **55**, 69-77.
- Miller, V.P., Stresser, D.M., Blanchard, A.P., Turner, S. & Crespi, C.L. (2000), Fluorometric high-throughput screening for inhibitors of cytochrome P450. *Annals of the New York Academy of Sciences* **919**, 26-32.
- Miyamoto, J. & Klein, W. (1998). Environmental exposure, species differences and risk assessment. *Pure and Applied Pharmacology* **70**, 1829-1845.
- Moore, L., Parks, D., Jones, S., Bleadsoe, R., Consler, T., Stimmel, J., Goodwin, B., Liddle, C., Blanchard, S., Willson, T., Collins, J., Kliewer, S. (2000). Orphan nuclear receptors Constitutive androstane receptor and Pregnane X Receptor share xenobiotic and steroid ligands. *J Biol Chem* **275**: 15122-15127, 2000.
- Morrell, S.L., Fuchs, J.A. & Holtzman, J.L. Effect of methoxychlor administration to male rats on hepatic, microsomal Iodothyronine 5'-Deiodinase, form I. *Journal Pharmacology Experimental Therapeutics* **294**, 308-312.
- Nagy, S.R., Sanborn, J.R., Hammock, B.D., & Denison, M.S. (2002). Development of a green fluorescent protein-based cell bioassay for the rapid and inexpensive detection and characterisation of Ah receptor agonists. *Toxicological Sciences*, **65**, 200-210.
- Nakajima, M., Yoshida, R., Shimada, N., Yamazaki, H. & Yokoi T. (2001). Inhibition and inactivation of human cytochrome P450 isoforms by phenethyl isothiocyanate. *Drug Metabolism and Disposition* **29**, 1110-1113.
- Nakano, S., Nagao, Y., Kobayashi, T., Tanaka, M., Hirano, S., Nobuhara, Y., & Yamada, T. (2002) Problems with Methods used to Screen Estrogenic Chemicals by Yeast Two-Hybrid Assays. *Journal of Health Science*, **48** (1) 83-88 (2002)
- Netzeva, T.I., Gallegos Saliner, A., Worth, A.P. (2006) Comparison of the applicability domain of a quantitative structure-activity relationship for estrogenicity with a large chemical inventory. *Environ Toxicol Chem.* **25(5)**:1223-30.
- Neubert, D. (1997). Vulnerability of the endocrine system to xenobiotic influence. *Regulatory Toxicology and Pharmacology* **26**, 9-29.
- Nilsson, S., Mäkelä, S., Treuter, E., Tujague, M., Thomsen, J., Andersson, G., Enmark, E., Petterson, K., Warner, M. & Gustafsson, J-A. (2001). Mechanisms of estrogen action. *Physiol. Rev.* **81**, 1535-1565.
- Nishimura M., Yaguti H., Yoshitsugu H., Naito S. & Satoh T. (2003). Tissue distribution of mRNA expression of human cytochrome P450 isoforms assessed by high sensitivity real-time reverse transcription PCR. *Yakugaku Zasshi*, **123**, 36-375.

Nishiyama T, Ogura K, Nakano H, Kaku T, Takahashi E, Ohkubo Y, Sekine K, Hiratsuka A, Kadota S, Watabe T. (2002) Sulfation of environmental estrogens by cytosolic human sulfotransferases. *Drug Metab Pharmacokinet.* 17(3):221-8.

OECD (1997) OECD Guidelines for the Testing of Chemicals – TG 471 Bacterial Reverse Mutation Test, available at: <http://www.sourceoecd.org>

Odum, J., Lefevre, P.A., Tittensor, S., Paton, D., Routledge, E.J., Beresford, N.A., Sumpter, J.P. & Ashby, J. (1997). The rodent uterotrophic assay: critical protocol features, studies with nonyl phenols, and comparison with a yeast estrogenicity assay. *Regulatory Toxicology and Pharmacology* **25**, 176-188.

Ohtake F., Takeyama K.-I., Matsumoto T., Kitagawa H., Yamamoto Y., Nohara K., Tohyama C., Krust A., Mimura J., Chambon P., Yanagisawa J., Fujii-Kuriyama Y. & Kato S. (2003) Modulation of oestrogen receptor signaling by association with the activated dioxin receptor. *Nature* **423**, 545-550.

Olsen, C., Meussen-Elholm, E., Hongslo, J., Stenersen, J., and Tollefsen, K. (2005). Estrogenic effects of environmental chemicals: an interspecies comparison. *Comparative Biochemistry and Physiology. Toxicology & Pharmacology* **141**, 267-274.

Orti, E. Bodwell, J.E. & Munck A. (1992). Phosphorylation of steroid hormone receptors. *Endocrine Reviews* **13**, 105-128.

Ozawa, S., Ohta, K., Miyajima, A., Kurebayashi, H., Sunouchi, M., Shimizu, M., Murayama, N., Matsumoto, Y., Fukuoka, M. & Ohno, Y. (2000). Metabolic activation of o-phenylphenol to a major cytotoxic metabolite, phenylhydroquinone: role of human CYP1A2 and rat CYP2C11/CYP2E1. *Xenobiotica* **30**, 1005-1017.

Ozers M.S., Ervin K.M., Steffen C.L., Fronczak J.A., Lebakken C.S., Carnahan K.A., Lowery R.G., Burke T.J. (2005) Analysis of ligand-dependent recruitment of coactivator peptides to estrogen receptor using fluorescence polarization. *Mol Endocrinol.* **19**:25-34.

Parke, D.V., Ioannides, C. & Lewis, D.F.V. (1990). The safety evaluation of drugs and chemicals by the use of computer optimised molecular parametric analysis of chemical toxicity (COMPACT). *ATLA* **18**, 91-102.

Pelkonen, O., Raunio, H., Rautio, A. and Lang, M. (1999): Chapter 8. Xenobiotic-metabolising enzymes and cancer risk: correspondence between genotype and phenotype. In: *Metabolic Polymorphisms and Susceptibility to Cancer* (Editors: Vineis, P., Malats, N., Lang, M., d'Errico, A., Caporaso, N., Cuzick, J. and Boffetta, P.) IARC Scientific Publications Nr. 148, pp 77-88.

Pendurthi, U.R., Lamb, J.G., Nguyen, N., Johnson, E.F. & Tukey R.H. (1990). Characterization of the CYP2C5 gene in 21L III/K rabbits. *Journal of Biological Chemistry*, **24**, 14662-14668.

Penning, T.M. (1997). Molecular endocrinology of hydroxysteroid dehydrogenases. *Endocr. Revs.* **18**, 281-305.

Philip, P.A., Ali-Sadat, S., Doehmer, J., Kocarek, T., Akhtar, A., Lu, H. & Chan, K.K. (1999). Use of V79 cells with stably transfected cytochrome P450 cDNAs in studying the metabolism and effects of cytotoxic drugs. *Cancer Chemotherapy and Pharmacology* **43**, 59-67.

Poellinger, L. (2000). Mechanistic aspects-the dioxin (aryl hydrocarbon) receptor. *Food Additives and Contaminants* **17**, 261-266.

- Purchase, I.F. (1999). Ethical review of regulatory toxicology guidelines involving experiments on animals: the example of endocrine disruptors. *Toxicological Sciences* **52**, 141-147.
- Ramamoorthy, K., Wang, F., Chen, I.-C., Norris, J.D., McDonnell, D.P., Leonard, L.S., Gaido, K.W., Bocchinfuso, W.P., Korach, K.S. & Safe, S. (1997). Estrogenic activity of a dieldrin/toxaphene mixture in the mouse uterus, MCF-7 human breast cancer cells and yeast-based estrogen receptor assays: no apparent synergism. *Endocrinology* **138**, 1520-1527.
- Rietjens, I. M. C. M., Boersma, M. G., van der Woude, H., Jeurissen, S. M. F., Schutte, M. E., and Alink, G. M. (2005). Flavonoids and alkenylbenzenes: Mechanisms of mutagenic action and carcinogenic risk: *Genes and Environment. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis* **574**, 124-138.
- Rogers, J.M. & Denison M.S. (2000). Recombinant cell bioassays for endocrine disruptors: Development of a stably transfected human ovarian cell line for the detection of estrogenic and anti-estrogenic chemicals. *In Vitro and Molecular Toxicology* **13**, 1, 67-82.
- Rogers J.M. & Denison M.S. (2002). Analysis of the antiestrogenic activity of 2,3,7,8-tetrachlorodibenzo-p-dioxin in human ovarian carcinoma BG-1 cells. *Molecular Pharmacology*, **61**, 1393-1403.
- Rose, P., Faulkner, K., Williamson, G. & Mithen, R. (2000). 7-Methylsulfinylheptyl and 8-methylsulfinyloctyl isothiocyanates from watercress are potent inducers of phase II enzymes. *Carcinogenesis* **21**, 1983-1988.
- Ross, M. K., and Pegram, R. A. (2004). *In vitro* biotransformation and genotoxicity of the drinking water disinfection byproduct bromodichloromethane: DNA binding mediated by glutathione transferase theta 1-1. *Toxicology and Applied Pharmacology* **195**, 166-181.
- Rouleau N., Turcotte S., Mondou M.H., Roby P., Bosse R. (2003) Development of a versatile platform for nuclear receptor screening using AlphaScreen. *J Biomol Screen.* **8**:191-7.
- Roy, D., Angelini, N.L. & Belsham D.D. (1999). Estrogen directly represses gonadotropin-releasing hormone (GnRH) gene expression in estrogen receptor- α (ER α)- and ER β -expressing GT1-7 GnRH neurons. *Endocrinology* **140**: 5045-5053.
- Saarikoski, S.T., Rivera, S.P., Hankinson, O., and Husgafvel-Pursiainen, K. (2005): CYP2S1: A short review. *Toxicol. And Appl. Pharmacol* **207**, S62-S69.
- Saeki, M., Saito, Y., Nagano, M., Teshima, R., Ozawa, S., and Sawada, J. (2002): mRNA expression of multiple cytochrome P450 isozymes in four types of cultured skin cells. *International Archives of Allergy and Immunochemistry* **127**, 333-336.
- Safe, S. (2000). Endocrine disruptors and human health - Is there a problem? An update. *Environmental Health Perspectives* **108**, 487-493.
- Safe, S., Connor, K., Ramamorthy, K., Gaido, K. & Maness, S. (1997). Human exposure to endocrine-active chemicals: hazard assessment problems. *Regulatory Toxicology and Pharmacology* **26**, 52-58.

Sakaki, T., Akiyoshi-Shibata, M., Murakami, H. & Ohkawa, H. (1991). Progesterone metabolism in recombinant yeast simultaneously expressing bovine cytochromes P450c17 (CYP 17A1) and P450c21 (CYP 21B1) and yeast NADPH-P450 oxidoreductase. *Pharmacogenetics* **1**, 86-93.

Sanderson, J.T., Slobbe, L., Lansbergen, G.W.A., Safe, S., & van den Berg, M. (2001). 2,3,7,8-Tetrachlorodibenzo-p-dioxin and diindolylmethanes differentially induce cytochrome P450 1A1, 1B1, and 19 in H295R human adrenocortical carcinoma cells. *Toxicological Sciences* **61**, 40-48.

Schenk, D., Stresser, D.M., McCants, J.C., Nimrod, A.C. & Benson, W.H. (1997). Influence of β -naphthoflavone and methoxychlor pretreatment on the biotransformation and estrogenic activity of methoxychlor in Channel Catfish (*Ictalurus punctatus*). *Toxicology and Applied Pharmacology* **145**, 349-356.

Schmalix, W.A., Lang, D., Schneider A., Bocker, R., Greim, H. & Doehmer, J. (1996). Stable expression and coexpression of human cytochrome P450 oxidoreductase and cytochrome P450 1A2 in V79 Chinese hamster cells: sensitivity to quinones and biotransformation of 7-alkoxyresorufins and triazines. *Drug Metabolism and Disposition* **24**, 1314-1319.

Schmieder, P., Tapper, M., Denny, J., Kolanczyk, R., Sheedy, B., Henry, T., and Veith, G. (2004). Use of trout liver slices to enhance mechanistic interpretation of ER binding for cost-effective prioritization of chemicals within large inventories. *Environ Sci Tech.* **38**, 6333-6342.

Schmieder, P., M. Tapper, A. Linnun, J. Denny, R. Kolanczyk and R. Johnson. 2000. Optimization of a Precision-Cut Trout Liver Tissue Slice Assay as a Screen for Vitellogenin Induction: Comparison of Slice Incubation Techniques. *Aquatic Toxicol.* **49**:251-268.

Schmitmeier, S., Langsch, A., Jasmund, I., Bader, A. (2006) Development and characterisation of a small-scale bioreactor based on a bioartificial hepatic culture model for predictive pharmacological *in vitro* screenings. *Biotechnol Bioeng.* **95(6)**:1198-206

Schmutzler, C., Bacinski, A., Gotthardt, I., Huhne, K., Ambrugger, P., Klammer, H., Schlecht, C., Hoang-Vu, C., Gruters, A., Wuttke, W., Jarry, H., Köhrle, J. (2007) The ultraviolet filter benzophenone 2 interferes with the thyroid hormone axis in rats and is a potent *in vitro* inhibitor of human recombinant thyroid peroxidase. *Endocrinology.* **148(6)**:2835-44.

Schrader, T.J., Langlois, I., Soper, K., & Cherry, W. (2002). Mutagenicity of bisphenol A (4,4'-isopropylidenediphenol) *in vitro*: effects of nitrosylation. *Teratogenesis Carcinogen Mutagen* **22**:425-441.

Seidel, S.D., Li, V., Winter, G.M., Rogers, W.J., Martinez, E.I., & Denison, M.S. (2000). Ah receptor-based chemical screening bioassays: application and limitations for the detection of Ah receptor agonists. *Toxicological Sciences* **55**, 107-115.

Setchell, K.D.R., Borriello, S.P., Hulme, P., Kirk, D.N., & Axelson, M. (1984). Nonsteroidal estrogens of dietary origin: possible roles in hormone-dependent disease. *Am J Clin Nutr.* **40**, 569-578.

Setchell, K.D.R., Brown, N.B., Zimmer-Nechemias, L., Brashear, W.T., Wolfe, B.E., Kirschner, A.S., & Heubi, J.E. (2002). Evidence for lack of absorption of soy isoflavone glycosides in humans, supporting the crucial role of intestinal metabolism for bioavailability. *Am J Clin Nutr* **76**, 447-453.

- Setchell, K.D.R., Clerici, C., Lephart, D., Cole, S.J., Heenan, C., Castellani, D., Wolfe, B.E., Nechemias-Zimmer, L., Brown, N.M., Lund, T.D., Handa, R.J., & Heubi, J.E. (2005). S-Equol, a potent ligand for estrogen receptor β , is the exclusive enantiomeric form of the soy isoflavone metabolite produced by human intestinal bacterial flora. *Am J Clin Nutr.* **81**,1072-1079.
- Shelby, M.D., Newbold, R.R., Tully, D.B., Chae, K. & Davis, V.L. (1996). Assessing environmental chemicals for estrogenicity using a combination of *in vitro* and *in vivo* assays. *Environmental Health Perspectives* **104**, 1296-1300.
- Sheets, P.L., Yost, G.S. and Carlson, G.P. (2004). Benzene metabolism in human lung cell lines BEAS-2B and A549 and cells overexpressing CYP2F1. *J. Biochem Mol Toxicol* **18**, 92-99.
- Shevtsov, S., Petrotchenko, V., Pedersen, L.C. & Negishi, M. (2003). Crystallographic analysis of a hydroxylated polychlorinated biphenyl (OH-PCB) bound to catalytic estrogen binding site of human estrogen sulfotransferase. *Environmental Health Perspectives* **7**, 884-888.
- Shilling, A., & Williams, D. (2000). Determining relative estrogenicity by quantifying vitellogenin induction in rainbow trout liver slices. *Toxicol Applied Pharmacol* **164**, 330-335.
- Shiple, J.M., & Waxman, D.J. (2006) Aryl hydrocarbon receptor-independent activation of estrogen receptor-dependent transcription by 3-methylcholanthrene. *Toxicol Appl Pharmacol.* **1;213(2):87-97**.
- Shou, M., Korzekwa, K.R., Brooks, E.N., Krausz, K.W., Gonzalez, F.J. & Gelboin, H.V. (1997). Role of human hepatic cytochrome P450 1A2 and 3A4 in the metabolic activation of estrone. *Carcinogenesis* **18**, 207-214.
- Sievernich A., Wildt, L., Lichtenberg-Fraté H. (2004) *In vitro* bioactivity of 17 α -estradiol. *Journal of Steroid Biochemistry and Molecular Biology.* **92**, 455-463.
- Sivaraman A., Leach J.K., Townsend S., Iida T., Hogan B.J., Stolz D.B., Fry R., Samson L.D., Tannenbaum S.R., Griffith L.G. (2005) A microscale *in vitro* physiological model of the liver: predictive screens for drug metabolism and enzyme induction. *Curr Drug Metab.* **6:569-91**.
- Smith, D.J. (1998). The pharmacokinetics, metabolism, and tissue residues of β -adrenergic agonists in livestock. *J. Anim. Sci.* **76**, 173-194.
- Smith G, Wolf CR, Deeni YY, Dawe RS, Evans AT, Comrie MM, Ferguson J, Ibbotson SH. (2003) Cutaneous expression of cytochrome P450 CYP2S1: individuality in regulation by therapeutic agents for psoriasis and other skin diseases. *Lancet.* **361(9366):1336-43**.
- Snyder, R.W., Maness, S.C., Gaido, K.W., Welsch, F., Sumner, S.C.J. and Fenell, T.R. 2000. Metabolism and disposition of bisphenol A in female rats. *Toxicol. Appl. Pharmacol.* **168**: 225-234.
- Soprano, D.R. & Soprano, K.J. (2003). Pharmacological doses of some synthetic retinoids can modulate both the aryl hydrocarbon receptor and retinoid pathways. *J. Nutr.* **133**, 277S-281S.
- Soto, A.M. & Sonnenschein, C. (2001). The two faces of Janus: sex steroids as mediators of both cell proliferation and cell death. *J. Natl. Cancer Institute* **93**, 1673-1675.

Spiegelhalder, B., Eisenbrand, G. & Presussmann, R. (1976). Influence of dietary nitrate on nitrite content of human saliva: possible relevance to *in vivo* formation of N-nitroso compounds. *Food Cosmet Toxicol.* **14**,545-548.

Spink, D.C., Lincoln II, D.W., Dickerman, H.W. & Gierthy, J.F. (1990). 2,3,7,8-Tetrachlorodibenzo-p-dioxin causes an extensive alteration of 17 β -estradiol metabolism in MCF-7 breast tumor cells. *PNAS* **87**, 6917-6921.

Stanley LA, Skare JA, Doyle E, Powrie R, D'Angelo D, & Elcombe CR. Lack of evidence for metabolism of p-phenylenediamine by human hepatic cytochrome P450 enzymes. *Toxicology*. 2005; 210(2-3):147-57

Steimer, T. (2003). *Steroid Hormone Metabolism*. Geneva Foundation for Medical Research.
http://www.gfmer.ch/Books/Reproductive_health/Steroid_hormone_metabolism.html

Stresser, D. & Kupfer, D. (1997). Catalytic characteristics of CYP3A4: Requirement for phenolic function in *ortho* hydroxylation of estradiol and *O*-demethylated methoxychlor. *Biochemistry* **36**, 2203-2210.

Stresser, D.M. & Kupfer, D. (1998a). Human cytochrome P450 catalysed conversion of the prestrogenic pesticide methoxychlor into an estrogen: Role of CYP2C19 and 1A2 in *O*-demethylation. *Drug Metab. Dispos.* **26**, 868-874.

Stresser, D.M. & Kupfer, D. (1998b). Prosubstrates of CYP3A4, the major human hepatic cytochrome P450. *Biochem. Pharmacol.* **55**, 1861-1871.

Stromstedt, M., Warner, M., Banner, C.D., Macdonald, P.C. & Gustafsson, J.A. (1993). Role of brain cytochrome P450 in regulation of the level of anesthetic steroids in the brain. *Mol. Pharmacol.* **44**, 1077-1083.

Suiko, M., Sakakibara, Y., and Liu, M.C. 2000. Sulfation of environmental estrogen-like chemicals by human cytosolic sulfotransferases. *Biochem. Biophys. Res. Commun.* 267: 80-84.

Swales, K. (2002) An investigation of host cell effects on the xenobiotic induction of cytochrome P450 3A. PhD thesis. University of Surrey, UK

Talafous, J., Sayre, L.M., Mieyal, J.J. et al. (1994). META. 2. A dictionary model of mammalian xenobiotic metabolism. *Journal of Chemical Information and Computer Sciences* **34**, 1326-1333.

Tang, M. H. Y., Helsby, N. A., Wilson, W. R., and Tingle, M. D. (2005). Aerobic 2- and 4-nitroreduction of CB 1954 by human liver. *Toxicology* **216**, 129-139.

Tannenbaum S.R., Weisman M. & Fett D. (1975). The effect of nitrate intake on nitrite formation in human saliva. *Food Cosmet Toxicol* **14**,549-552.

Tew, K.D., Manks, A., Barone, L., Rosser, D., Akerman, G., Montali, J.A., Wheatley, J.B. & Schmidt, D.E. Jr. (1996): Glutathione-associated enzymes in human cell lines of the National Cancer Institute drug screening program. *Mol. Pharmacol.* **50**, 149-159.

Tucker, G.T., Houston, J.B. & Huang, S.M. (2001). Optimizing drug development: strategies to assess drug metabolism/transporter interaction potential-towards a consensus. *Br J Clin Pharmacol.* **52**, 107-117.

Tominaga, T., Negishi, T., Hiroka, H., Miyachi, A., Inoue, A., Hayasaka, I., and Yoshikawa, Y. 2006. Toxicokinetics of bisphenol A in rats, monkeys and chimpanzees by the LC-MS/MS method. *Toxicology* 226, 208-217.

Turan, N. Waring, R.H., & Ramsden, D.B. (2005) The effect of plasticisers on "sulphate supply" enzymes. *Mol Cell Endocrinol.* **1;244(1-2)**:15-9.

UNEP (2004) Stockholm Convention on Persistent Organic Pollutants, United Nations Environment Programme (UNEP). Available at: [<http://www.pops.int/>]

Vakharia, D. & Gierthy, J. (1999). Rapid assay for oestrogen receptor binding to PCB metabolites. *Toxicology in vitro* **13**, 275-282.

van Lipzig, M.M., Commandeur, J.N., de Kanter, F.J., Damsten, M.C., Vermeulen, N.P., Maat, E., Groot, E.J., Brouwer, A., Kester, M.H., Visser, T.J., Meerman, J.H. (2005). Bioactivation of dibrominated biphenyls by cytochrome P450 activity to metabolites with estrogenic activity and estrogen sulfotransferase inhibition capacity. *Chem Res Toxicol.* **18(11)**:1691-700

Vedani, A., Dobler, M. & Lill, M.A. (2005). In silico prediction of harmful effects triggered by drugs and chemicals. *Toxicol. Appl. Pharmacol.* **207** suppl. 2, 398-407.

Völkel, W., Bittner, N., & Dekant, W. (2005) Quantitation of bisphenol A and bisphenol A glucuronide in biological samples by high performance liquid chromatography-tandem mass spectrometry. *Drug Metab Dispos.* **33(11)**:1748-57.

Warner, M., Hellmold, H., Yoshida, S., Liao, D., Hedlund, E. & Gustafsson, J.A. (1997). Cytochrome P450 in the breast and brain: role in tissue-specific activation of xenobiotics. *Mutation Res.*, **376**, 79-85.

Warner, M., Hellmold, H., Magnusson, M., Rylander, T., Hedlund, E. & Gustafsson, J.A. (1998). Extrahepatic cytochrome P450: role in in situ toxicity and cell-specific hormone sensitivity. *Arch. Toxicol. Suppl.* **20**, 455-463.

Warner, M., Nilsson, S. & Gustafsson, J-Å. (1999). The estrogen receptor family. *Curr. Opin. Obstet. Gynecol.* **11**, 249-254.

Waters, K.M., Safe, S. & Gaido, K.W. (2001). Differential gene expression in response to methoxychlor and estradiol through ER α , ER β , and AR in reproductive tissues of female mice. *Toxicological Sci.* **63**, 47-56.

Waxman, D.J., (1988) Interactions of hepatic cytochromes P-450 with steroid hormones. Regioselectivity and stereospecificity of steroid metabolism and hormonal regulation of rat P-450 enzyme expression. *Biochem Pharmacol.* **1;37(1)**:71-84.

Waxman, DJ., (1999) P450 gene induction by structurally diverse xenochemicals: central role of nuclear receptors CAR, PXR and PPAR. *Arch Biochem Biophys* **369**: 11-23.

Wei, P., Zhang, J., Egan-Hafley, M., Liang, S. & Moore, D.D. (2000). The nuclear receptor CAR mediates specific xenobiotic induction of drug metabolism. *Nature*, **407**, 920-923.

Weigel, N. (1996). Steroid hormone receptors and their regulation by phosphorylation. *Biochem. J.* **319**, 657-667.

Weybridge Conference (1996). European workshop on the impact of endocrine disrupters on human health and wildlife. Report of proceedings, EUR 17549, DG XII of the European Commission.

Wilson, N.K., Chuang, J.C., Lyu, C., Menton, R. and Morgan, M.K. 2003. Aggregate exposures of nine preschool children to persistent organic pollutants at day care and at home. *J. Expos. Anal. Environ. Epidemiol.* **13**, 187-202.

Wilson, N.K., Chuang, J.C., Morgan, M.K., Lordo, R.A. and Sheldon, L.S. 2007. Exposures of preschool children to pentachlorophenol, bisphenol A and nonyl-phenol at home and daycare. *Environ. Res.* **103**, 9-20.

Wolf, C. R., Berry, P. N., Nash, J. A., Green, T., and Lock, E. A. (1984). Role of microsomal and cytosolic glutathione S-transferases in the conjugation of hexachloro-1:3-butadiene and its possible relevance to toxicity. *The Journal of Pharmacology and Experimental Therapeutics* **228**, 202-208.

Wolf, C.R. & Smith, G. (1999). Pharmacogenetics. *British Medical Bulletin* **55**, 366-368.

Wong, C., Kelce, W.R., Sar, M., & Wilson, E.M. (1995). Androgen receptor antagonist versus agonist activities of the fungicide vinclozolin relative to hydroxyflutamide. *Journal of Biological Chemistry* **270**, 19998-20003.

Woo, W-T., Arcos, J.C., & Lai, D.Y. (1988) In: P. Politzer & F.J. Martin Jr eds., *Chemical Carcinogens, Activation Mechanisms, Structural and Electronic Factors and Reactivity*, Elsevier, Amsterdam, pp. 1-31.

Wormhoudt, L.W., Commandeur, J.N. & Vermeulen, N.P. (1999). Genetic Polymorphisms of Human N-Acetyltransferase, Cytochrome P450, Glutathione-S-Transferase, and Epoxide Hydrolase Enzymes: Relevance to Xenobiotic Metabolism and Toxicity. *Crit. Rev. Toxicol.* **29** (1), 59-124.

Xu, C., Li, C.Y., & Kong, A.N. (2005). Induction of phase I, II and III drug metabolism/transport by xenobiotics. *Arch Pharm Res.* **28**, 249-268.

Yen P.M. (2001). Physiological and molecular basis of thyroid hormone action. *Physiological Reviews*, **81**, 1097-1142.

Yengi, L.G., Xiang, Q., Pan, J., Scatina, J., Kao, J., Ball, S.E., Fruncillo, R., Ferron, G., and Wolf, C.R. (2003): Quantitation of cytochrome P450 mRNA levels in human skin. *Anal. Biochem* **316**, 103-110.

Yoshihara, S., Makishima, M., Suzuki, N. & Ohta, S. (2001). Metabolic activation of bisphenol A by rat liver S9 fraction. *Toxicological Sciences* **62**, 221-227.

Yoshihara, S., Mizutare, T., Makishima, M., Suzuki, N., Fujimoto N., Igarashi, K. & Ohta, S. (2004) Potent estrogenic metabolites of bisphenol A and Bisphenol B formed by rat liver S9 fraction: their structures and estrogenic potency. *Toxicological Sciences* **78**, 50-59.

You, L. (2004). Steroid hormone biotransformation and xenobiotic induction of hepatic steroid metabolising enzymes. *Chem Biol Interact.* **147**, 233-246.

Zacharewski, T.R., Berhane, K., Gillesby, B.E. & Burnison, B.K. (1995). Detection of estrogen- and dioxin-like activity in pulp and paper mill black liquor and effluent using *in vitro* recombinant receptor/reporter gene assays. *Environmental Science & Technology* **29**, 2140-2146.

Zhou, L.-X., Dehal, S.S., Kupfer, D., Morrell, S., McKenzie, B.A., Eccleston Jr., E.D. & Holtzman, J.L. (1995). Cytochrome P450 catalysed covalent binding of methoxychlor to rat hepatic, microsomal iodothyronine 5'-monodeiodinase, type I; does exposure to methoxychlor disrupt thyroid hormone metabolism? *Arch. Biochem. Biophys.* **322**, 390-394.

Zhou, T., Ross, D.G., DeVito, M.J., & Crofton, K.M. (2001). Effects of short-term *in vivo* exposure to polybrominated diphenyl ethers on thyroid hormones and hepatic enzyme activities in weanling rats. *Toxicological Sciences* **61**, 76-82.

Ziccardi, M.H., Gardner, I.A., & Denison, M.S. (2000). Development and modification of a recombinant cell bioassay to directly detect halogenated and polycyclic aromatic hydrocarbons in serum. *Toxicological Sciences* **54**, 183-193.

7. TABLES

Table 1
EXAMPLES OF CELL LINES AND OTHER CELL TYPES USED IN CELL PROLIFERATION AND TRANSCRIPTIONAL ACTIVATION ASSAYS FOR TESTING ENDOCRINE DISRUPTORS (after Combes, 2000 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 (Swales, 2002)
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
EXAMPLES OF METABOLISM CONVERTING THE ENDOCRINE ACTIVITY OF SELECTED CHEMICALS.

Substance	Metabolite	Change in activity	Reference
Tamoxifen	4-OH derivative	↑ anti-estrogenic activity	Cassidy & Milligan 1998
Chlorobiphenyl	2-chloro-4-hydroxy-biphenyl	↑ estrogenic activity	Rosenkranz unpublished
PCBs	Aryl sulfone	↑ anti-estrogenic activity	Letcher, et al., 2002
	Methyl sulfonyl	↑ glucocorticoid receptor interaction	Johansson, et al., 1998; 2002
Vinclozolin	2-[[3,5-dichlorophenyl-carbamoyl]oxy]-2-methyl-3butenoic acid (M1)	↓ anti-estrogenic activity	Wong, et al., 1995
	3',5'-dichloro-2-hydroxy-2-methylbut-3-enamide (M2)	↑ anti-estrogenic activity	

Table 3.
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
3A4/5 (human)	Dexamethasone, Testosterone	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 4.***THE ED/EAS TEST METHODS CURRENTLY IN (PRE)VALIDATION, UNDER THE AUSPICES OF THE OECD.***

Receptor Binding Assays				
hrER α	The Freyburger Wilson Assay (FWA) assay protocol utilises the Pan Vera hrER α full length ER, and the CERI protocol utilizes the CERI-ER α , which contains the ligand binding domain of hrER α .	binding	Validation starting in June 2007 in 5 labs.	US lead (US EPA) international collaboration study (EC ECVAM; CERI, Japan)
hrAR	Human recombinant AR assay. Ligand binding domain expressed in E. coli.	binding	Under development. Approximately 900 chemicals have been tested.	METI, Japan
rAR	Rat recombinant AR assay. (ligand binding domain is identical to human LBD).	binding	Some limited prevalidation work has been conducted in the EU funded project ReProTect. Options are currently being explored.	Lead and international collaboration work under discussion
hrTR	Human recombinant TR assay. Full-length expressed in E. coli. TRs α 1 and β 1 binding assays.	binding	Under development. Approximately 60 chemicals have been tested using both receptors.	METI, Japan
Transcriptional Activation Assays				
	HeLa-9903 cells with plasmids containing hER α cDNA driven by SV40 promotor and luciferase reporter plasmid.	Stable, ag/antag	The agonist assay was peer reviewed in March-07. International validation	CERI/MHLW, Japan

ER α			of the antagonist assay is planned for 2007.	
	HeLa-9903 cells: hER α /pcDNA3.1 receptor expressing plasmid and ERE-AUG-Luc+ reporter plasmid	Transient, ag	Peer reviewed April 2007. The agonist assay draft TG will be proposed to WNT20 for adoption. SPSF submitted. Antagonist assay validation will start in 2008.	CERI/MHLW, Japan
	MELN. MCF-7 cells with endogenous ER α + luciferase stably transfected	ag/antag	Prevalidation in 2007. Report early 2008.	EC/ECVAM
	ER-CALUX. T47 D (human breast cancer) cells with endogenous ER α + luciferase stably transfected	ag/antag	Going through optimisation. Validation planned for 2008.	EC/ECVAM
	LUMI cell, BG1 cells with endogenous ER α + luciferase stably transfected (XDS Inc)	ag/antag	Validation initiated in late 2007, completed by late 2008.	US lead (ICCVAM) international collaboration study with ECVAM and JaCVAM
ER β	HeLa, hER β /pcDNA3.1, ERE-AUG-Luc+	Transient, ag	Completed data collection for 250 compounds	CERI/MHLW, Japan
AR	CV-1 cells hAR/pcDNA3.1 receptor expressing plasmid and ARE-AUG-Luc+ reporter plasmid	Transient, ag/antag	Prevalidated and validated in Japan in 4 labs, with 5 chemicals. Should be considered for (preliminary) Peer review.	CERI/MHLW, Japan
	AR-Ecoscreen TM stable CHO clone	Stable, ag/antag	Prevalidated and validated in Japan in 4	CERI/MHLW, Japan

			labs, with 5 chemicals. Peer reviewed early 2007.	
	PALM. PC-3 (prostate adenocarcinoma) cells stably transfected with hAR and luciferase reporter gene	ag/antag	Prevalidation in 2007/8	EC/ECVAM
	CALUX. U2-OS (bone cell) cells stably transfected with hAR and luciferase reporter construct	ag/antag	Prevalidation in 2007/8	EC/ECVAM
TR β	RXR co-transfected CHO cells are used	Transient, ag/antag	Under development, 150 chemicals tested so far.	MHLW, Japan
Aromatase & Steroidogenesis Assays				
	Microsomal aromatase assay, KGN cells		Prevalidated. Peer review report available in early 2008.	
	Steroidogenesis, H295R cell based assay		Validation and peer review by December 2008. SPSF submitted.	US lead international collaboration study

Table 5
SELECTED EXAMPLES OF METABOLISM OF STEROID HORMONES & EAS

<i>Substrate(s)</i>	<i>System</i>	<i>Observation</i>	<i>Reference</i>
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	6 β - hydroxylation	Gebhardt, et al., 1999
	Adult and foetal human brain fractions	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 & Roy, 1995
Estrone	Liver microsomes from 14 human organ donors	All samples metabolised estrone	Shou, et al., 1997

		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	
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 6
SYSTEMS THAT CAN BE USED FOR THE TESTING OF METABOLISM IN VITRO: A TIERED APPROACH

Strategies	Test systems	Endpoints	Applicability	Formal Status
First tier	Microsomes from human hepatocytes or from genetically engineered cell lines expressing human genes	Identification of metabolite formation by LC-MS/MS	Most important metabolic pathways	Prevalidation studies to be initiated
	Cell lines, primary monolayer cultures, genetically engineered cell lines expressing human genes	Cell morphology, viability, membrane damage, liver-specific endpoints, genotoxic endpoints	Metabolism-mediated toxic effects	
	Microsomes, human hepatocytes, genetically engineered cell lines expressing human genes	Quantification by LC-MS of the amount of the parent compound that remains after metabolism	Metabolic stability	
	Microsomes, human hepatocytes, genetically engineered cell lines expressing human genes	Quantification by LC-MS, HPLC or fluorescence	Inhibition	
Second tier	Short-term and long-term hepatocyte cultures (e.g. human hepatocyte sandwich cultures), hepatocytes generated by stem cell technology, precision-cut liver slices and liver-derived cell lines expressing or re-expressing biotransformation enzymes, highly differentiated human cell lines	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	Induction	Prevalidation study to be initiated

		chain reaction		
Third tier	Genetically engineered cell lines expressing human genes	Quantification by LC-MS	Polymorphism	Prevalidation study to be initiated

Table 7
EXAMPLES OF TYPICAL ACTIVITY ASSAYS AND AVAILABILITY OF COMMERCIALY AVAILABLE ANTIBODIES IN ORDER TO ANALYSE XENOBIOTIC-METABOLISING CYP ENZYMES.

	Activity Assay	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 Pentoxyresorufin O-depentyase 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

8. FIGURES

Figure 1. P450 Transcription factors (Jacobs, 2004, reprinted from Toxicology with permission from Elsevier.)

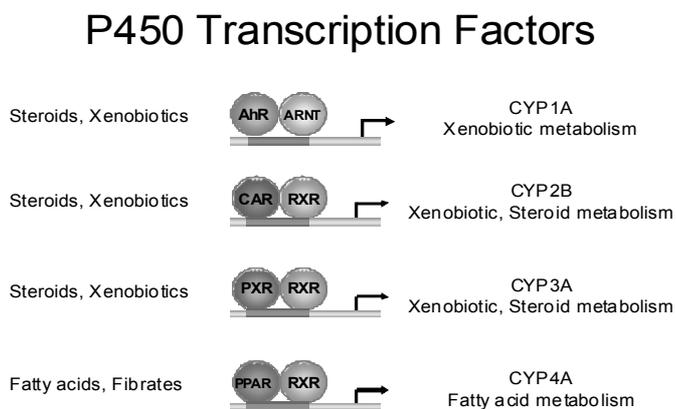


Figure 2. Key receptors and P450 in the steroidogenic pathway (Jacobs, 2004 reprinted from Toxicology with permission from Elsevier.)

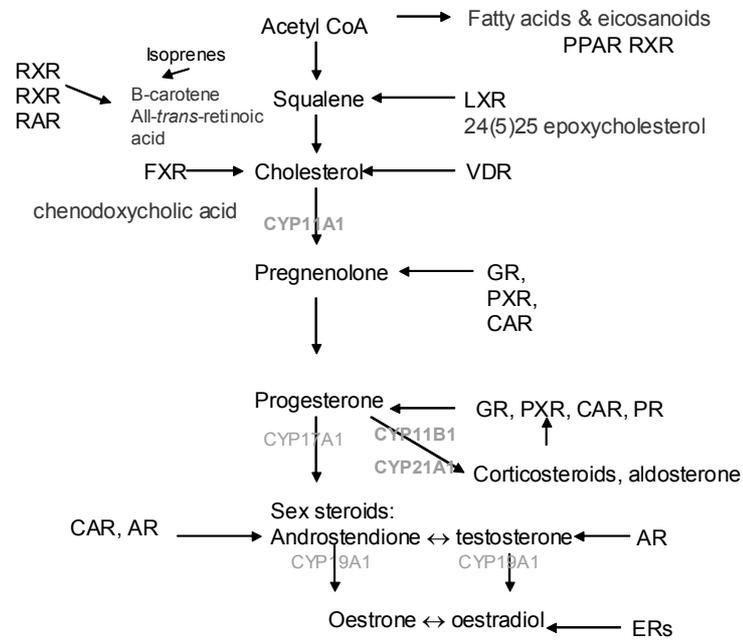


Figure 3. The synthesis of different steroid hormones with indications of the roles played by some P450s (Mensah-Nyagan et al., 1999 reprinted from Pharmacological Reviews with permission from the American Society for Pharmacology and Experimental Therapeutics.)

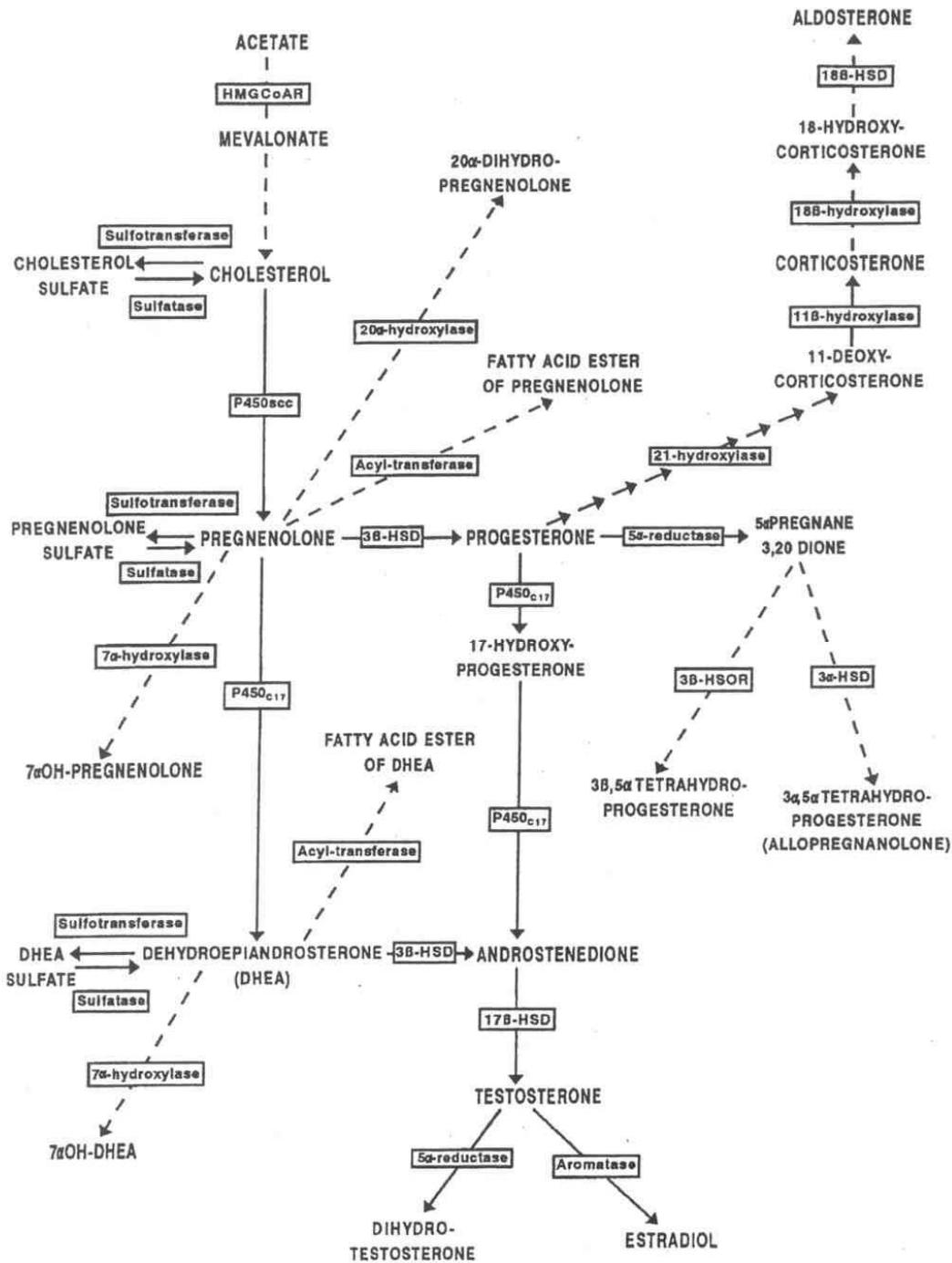


Figure 4. A simplified metabolic scheme of Methoxychlor (adapted from Hu and Kupfer, 2002a)

