

Metabolism: A Bottleneck in *In Vitro* Toxicological Test Development

The Report and Recommendations of ECVAM Workshop 54¹

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Preface

This is the 54th report of a series of workshops organised by the European Centre for the Validation of Alternative Methods (ECVAM). The main objective of ECVAM, as defined in 1993 by its Scientific Advisory Committee, is to promote the scientific and regulatory acceptance of alternative methods which are of importance to the biosciences, and which *reduce*, *refine* or *replace* the use of laboratory animals. One of the first priorities set by ECVAM was the implementation of procedures that would enable it to become well informed about the

state-of-the-art of non-animal test development and validation, and of opportunities for the possible incorporation of alternative methods into regulatory procedures. It was decided that this would be best achieved through a programme of ECVAM workshops, each addressing a specific topic, and at which selected groups of independent international experts would review the current status of various types of *in vitro* tests and their potential uses, and make recommendations about the best ways forward.

A workshop on *Metabolism: a bottleneck in in vitro toxicological test development*, was held at ECVAM on 26–29 January 2004, with participants

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¹This document represents the agreed report of the authors as individual scientists.

from academia, national organisations, regulatory bodies and industry. The aim of the workshop was to discuss the use of metabolising systems in *in vitro* toxicity test methods (i.e. the need to take account of biotransformation, and the methods available), to give some examples of how chemicals can be screened and tested for their susceptibilities to metabolic activation, and to consider how such information can also be used, in conjunction with computer modelling approaches, for providing information for use in hazard identification and risk assessment. In addition to plenary sessions, the workshop was organised into three working groups, to discuss: a) biokinetics and integration of techniques; b) metabolism and methodology; and c) target organ and target system toxicity. Some recommendations are listed at the end of this report, and these should be considered for future implementation, to encourage the incorporation of biotransformation into *in vitro* toxicity test methods.

Introduction

The nature of biotransformation

Biotransformation is the process whereby a substance, usually a foreign compound (xenobiotic), is chemically transformed in the body to form a metabolite or a variety of metabolites. This term will be used throughout this report to distinguish this process from other endogenous metabolic phenomena involved in controlling cellular functions, collectively known as *metabolism*.

Biotransformation is one of the main factors which influence the transport and partitioning of a chemical into and within the body, its toxicity, and its rate and route of elimination. All chemicals are potentially susceptible to biotransformation of some sort, and all cells and tissues are potentially capable of biotransforming xenobiotics, although the major sites of such biotransformation are the liver and the entry portals of the body (1). Species, strain, sex, individual, organ, and age-specific differences, and even differences between cells in the same organ, can have important effects on the biotransformation of xenobiotics. Exposure to drugs, occupational and industrial chemicals, or environmental pollutants, can lead to either the induction or the inhibition of biotransformation. Moreover, drug–drug, drug–toxicant and drug–food interactions, due to the induction and inhibition of specific biotransformation enzymes, are one of the most important factors which complicate drug therapy and cause adverse drug reactions.

The main phases of biotransformation

Biotransformation is usually divided into two main phases, phase I and phase II. Phase I is usually oxidative (e.g. hydrolysis, although reductive metabolism and non-redox reactions of functionalisation can also occur), and has a major protective function in rendering lipophilic molecules more polar and more readily excretable. In phase II, often referred to as detoxification, such oxidised moieties are subsequently conjugated with highly polar molecules, such as glucuronic acid, before they are excreted (Table 1). Phase I oxidation reactions are catalysed mainly by the many isoforms of the haeme-thiolate cytochrome P450 (CYP) supergene family of enzymes (the so-called mixed function oxidases [MFOs]), which collectively have very wide substrate specificity (2, 3). In humans and other mammalian species, the CYP1, CYP2 and CYP3 isozyme families are primarily associated with the phase I metabolism of exogenous compounds. Flavin monoamine oxidase (FMO) can also oxidise xenobiotics (4), although to a lesser extent than MFO enzymes. Phase II conjugation of the oxidised moiety with molecules such as glucuronic acid, sulphate, methionine, cysteine or glutathione, is catalysed by a variety of isozymes of N-acetyltransferase (NAT), uridine diphosphoglucuronosyl transferase (UGT), sulphotransferases (SULTs), and glutathione S-transferase (GST; 5). These enzymes, listed in Table 2, occur variously in the cytosol or are bound to microsomal proteins, and their structures and functions have been reviewed elsewhere (6).

Supergene families of isoforms of phase II enzymes also exist, which show different affinities and therefore selectivity toward different substrates. For example, the SULTs are a supergene family of cytosolic enzymes, for which some eleven distinct isoforms of the enzyme are known in humans, and these differ markedly in their catalytic activities for the same substrates and in their tissue locations (7; Table 2).

The practical aspects of studying CYP activities have been comprehensively reviewed by Phillips and Shephard (8), and general techniques for incorporating metabolism into *in vitro* toxicity assays have been described by Castell and Gomez-Lechon (9), Plant (10), Ekins (11), and in reference 12.

At least 50 major enzymes catalyse the biotransformation of xenobiotic chemicals, and many more are responsible for biotransforming at least a few chemicals. The interactions of chemicals with enzymes are characterised by affinity, turnover number, intrinsic activity, and, at a more general level, whether or not the reaction is rate-limiting in the metabolism of a given chemical, or a more minor one.

More recently, it has been suggested that a further, phase III, stage of metabolism should be recognised,

Table 1: A comparison of the main enzymes involved in biotransformation

Enzyme classes/enzymes	Types of drugs metabolised	Sources available/tissue distribution
Cytochrome P450 enzymes (about 13 “drug-metabolising” enzyme forms)	Practically 90% of all drug substances	Hepatocytes most versatile and comprehensive; other tissues and cell types, selectively
CYP1A1	Few pharmaceuticals, principally polycyclic aromatic hydrocarbons and other carcinogens	Recombinant enzyme; mainly extra-hepatic expression (e.g. placenta from smokers)
CYP3A4	A majority (> 50%) of all clinically used drugs	Hepatocytes (genetically engineered)
Flavin-monoxygenases (FMO; 5 forms)	Compounds with secondary and tertiary amines or sulphhydryl groups (chlorpromazine, desipramine, methimazole)	Hepatocytes most versatile and comprehensive
Prostaglandin H synthase (COX)	PAH-diols, aflatoxin B1, aromatic amines	Liver, kidney, bladder (microsomes)
Alcohol/aldehyde dehydrogenases and oxidases	Various compounds with alcohol and aldehyde functions (ethanol)	Many tissues (cytosol location)
Monoamine oxidase	Selegiline, moclobemide	Many tissues (mitochondrial location)
Esterases/hydrolases/peptidases	Compounds with cleavable ester/amide bond (procaine, succinylcholine, lidocaine)	Many tissues, including blood and blood cells
Reductases	Many substances with azo, nitro and carbonyl functions (chloramphenicol, naloxone); importance not well characterised	Present in hepatocytes (cytosol, some in microsomes)
UDP-glucuronosyl transferases; (UGT) glucuronide conjugation	Most drugs with suitable O-, S- and N-functional groups (morphine, diazepam, paracetamol)	Subcellular systems need UDPGA; hepatocytes most versatile and comprehensive (microsomes)
Sulphotransferases (SULT); sulphate conjugation	Phenols, alcohols, aromatic amines (paracetamol, methyl dopa)	Hepatocytes most versatile and comprehensive (cytosol); subcellular systems need 3-phosphoadenosine-5-phosphosulphate (PAPS)
GSH transferases (GST); glutathione conjugation	Epoxides, arene oxides, nitro groups, hydroxylamines (ethacrynic acid)	Hepatocytes most versatile and comprehensive (cytosol, microsomes)
Acyl-CoA glycinetransferase; amino acid conjugation	Acyl-CoA derivatives of carboxylic acids (salicylic acid)	Hepatocytes (mitochondria)
N-acetyltransferases (NAT); acylation	Amines (sulphonamides, isoniazid, clonazepam, dapsone)	Hepatocytes (cytosol)
Methyl transferases; methylation	Catecholamines, phenols, amines (L-dopa, thiouracil)	Various tissues (cytosol)

Table 2: Examples of drug metabolising enzymes available for incorporation into *in vitro* assays

Isoenzymes	Types of chemicals metabolised
Cytochrome P450 enzymes	Practically 90% of all chemical substances
CYP1A1	PAH
CYP1A2	Aromatic amines, amino acid pyrolysis products
CYP1B1	PAH, DMBA, oestrogens
CYP2A6	Nitrosamines, aflatoxins, nicotine, cotinine, butadien
CYP2B6	Cyclophosphamide, 6-aminochrysene
CYP2C8	Taxol
CYP2C9	NSAIDs, warfarin
CYP2C19	Proton pump inhibitors
CYP2D6	Alkaloids, one carcinogen 4-(methylnitrosamine)-1-(3-pyridyl)-1-butanone (NNK)
CYP2E1	Small molecular substances, solvents, benzene, styrene, nitrosamines
CYP3A4	A majority of drugs, aflatoxin B1, 1-nitropyrene
CYP3A5	Same as 3A4
CYP3A7	Same as 3A4
CYP4A9/11	Fatty acids
CYP4B	
Flavin-monooxygenases (FMO)	Oxidation of nucleophilic N, S and P heteroatoms of xenobiotics
FMO1	Chlorpromazine
FMO2	Nicotine
FMO3	Nicotine
FMO4	
FMO5	
Glucuronide conjugation	Most chemicals with suitable O-, S- and N-functional groups
UGT1A1	Bilirubin, catecholestrogens, opioids
UGT1A3	Amines, flavonoids, 7-hydroxycoumarins, opioids
UGT1A4	Amines, plant steroids, androgens, progestins
UGT1A6	Small planar phenols, paracetamol, p-nitrophenol
UGT1A7	Phenols, hydroxylated PAHs, coumarins
UGT1A8	Opioids, oestrogens, propofol, primary and secondary amines
UGT1A9	Large bulky phenols, propofol, primary and secondary amines, benzodiazepins, coumarins, flavonoids
UGT1A10	Mycophenoli acid, phenols, steroids, entacapone
UGT2B4	Hyodeoxycholic acid
UGT2B7	Opioids, steroids, aliphatic alcohols, PAHs, carboxylic acids
UGT2B15	Steroids, flavonoids
Soluble epoxide hydrolase	Epoxides
Microsomal epoxide hydrolase	Epoxides

relating to the excretion of conjugates and involving ATP-dependent plasma membrane transporters (for example, p-glycoprotein, MRP2, and organic anion transporting polypeptides [OATPs; 13]), which are involved in the cellular uptake of drugs.

The role of biotransformation in the activation of xenobiotics

The biotransformation of xenobiotics can result in the formation of highly reactive chemicals that are

known to play a role in toxicity, particularly in mutagenesis and carcinogenesis (a process known as bioactivation). However, there are numerous examples of a requirement for bioactivation for other toxicity endpoints and chemicals, including endocrine disruptors such as methoxychlor and vinclozolin, and neurotoxicants such as the organophosphorothionate (OPT) pesticides (14), and paracetamol, carbamazepine, tienylic acid and halothane. Also, the resulting metabolites might interact with enzymes involved in the biosynthesis of endogenous compounds, with receptors, or with

signal transduction mechanisms, so the generation of such metabolites may be important (e.g. in the case of endocrine disruption caused by steroidogenesis [14]).

During phase I reactions, in particular, highly reactive and potentially toxic intermediates can be generated. Much evidence exists for the bioactivation of many chemicals and drugs, and much of this report is concerned with such effects (for recent reviews, see 15, 16). However, phase II metabolism is also capable of activating chemicals to produce highly reactive electrophilic intermediates. For example, it is well established that acyl glucuronides, arising from the glucuronidation of a wide range of carboxylic acid substrates, can covalently bind to macromolecules; this could well lead to toxicity in humans following the administration of carboxylate drugs (16). Also, several 1,2-dihaloalkanes and 1,2-dihaloethanes, such as 1,2-dichloromethane, are susceptible to glutathione transferase-dependent bioactivation as a result of the formation of S-(halomethyl)glutathione and glutathione episulphonium ions as electrophiles (17). In addition, sulphotransferases can bioactivate several chemicals via *O*-sulphonation (for example, hydroxymethyl polycyclic aromatic hydrocarbons and N-hydroxy derivatives of carcinogenic arylamines and heterocyclic amines [18]). These strong electrophiles arise because the sulphate group is electron-withdrawing and is susceptible to cleavage from the rest of the molecule, leaving behind a highly charged cation (7). Also, epoxide hydrolases can activate certain molecules possessing epoxide groups, either formed as a result of phase I oxidation (for example, during the biotransformation of benzo[a]pyrene) or present in the parent compound (19). Chemicals which have reactive and potentially toxic conjugates include morphine, safrole, and certain nitro and chlorinated aromatic compounds. There is an important need to take account of the possible biotransformation of chemicals in *in vitro* assays, since the absence of this crucial determinant of toxicity could give rise to false-positive data (due to lack of detoxification) or to false-negative data (due to lack of activation).

Toxicity in whole animals can be due to the parent compound, to metabolites formed at the site of entry into the body (such as lung, gut and skin) and/or in the liver, and which are sufficiently stable to reach other target organs (systemic toxicity), or to metabolites formed in the target organ itself. Substances that are metabolised in, for example, the liver, may be subjected to further metabolism once they reach their target organs.

Many instances exist of metabolites generated at one site in the body and active at another target site. Several examples of this for carcinogenicity will be given: benzo[a]pyrene, β -naphthylamine, heterocyclic amines, trichloroethylene and ecstasy. After hepatic metabolism, benzo[a]pyrene binds to

macromolecules in distal perfused lungs (20). Also, arylamines, like β -naphthylamine, are directly detoxified by NAT isozymes NAT1 and NAT2 in the liver, leading to inactive amides. Alternatively, these arylamines can be bioactivated by CYP1A2, primarily to hydroxylated reactive products, which are inactivated by UDPGT-mediated conjugation, mainly with glucuronic acid. The arylamines can also be acetylated by NATs, although this is a less prominent activation route. The resulting hydroxy-arylamines can enter the blood system, generally as glucuronides and undergo renal filtration, arriving at the urinary bladder where they are deconjugated by β -glucuronidases to re-form their respective hydroxy-arylamines, the ultimate carcinogens. In individuals who are slow acetylators, much higher amounts of P450-mediated hydroxy-arylamines are formed, due to competition between NAT and CYP1A2 which is in favour of the latter. This increases the risk of bladder cancer in such individuals (21).

The heterocyclic amines are potent mutagens, formed during the cooking of meat and fish. In the liver, the major metabolic reaction is N-hydroxylation catalysed by hepatic CYPs, eventually leading to N-glucuronidation by UDPGT. The resulting N-hydroxy-glucuronides are transported via the bile to the colonic lumen. The colonic mucosa contains significant amounts of NATs, able to efficiently catalyse the *O*-acetylation of N-hydroxy derivatives to reactive N-acetoxyarylamines, which are responsible for DNA adduct formation and colon cancer. This outcome occurs more frequently in individuals who are fast acetylators (22).

Trichloroethylene is also activated in the liver before exerting its carcinogenicity in another tissue, in this case, the kidney. OPT pesticides are activated to toxic compounds in the liver and then inhibit AChE in the blood or brain, although a local cerebral metabolic activation step might also occur (23, 24). Lastly, ecstasy exerts its serotonergic neurotoxicity as a result of its conjugated metabolites, the formation of which is initiated systemically (25).

Biotransformation and toxicokinetics

Modulation of transport

The metabolic stability of a molecule and the rate of its biotransformation can affect the potential for its bioaccumulation, and whether it is likely to become more or less toxic, without it necessarily being converted into a more reactive metabolite. For example, topically applied substances can be rendered more absorbable and therefore potentially more toxic by being metabolised in the skin, so that they pass through it to exert their inherent toxicity

locally and/or systemically (26, 27). These processes can be quantified during toxicokinetic investigations.

However, the ability of the metabolites of a chemical that have been formed as a result of biotransformation at some site in the body, to induce toxicity in a target organ elsewhere, is also dependent on the ability of the metabolites to be transported to that target organ and to enter it. These phenomena are compound-dependent, and can also depend on the presence of the correct transporters, the permeability of membranes to passive diffusion of reactive intermediates, and/or the stereo-isomeric form of the substrate, due to the stereoselectivity of biotransformation enzymes (28).

The blood-brain, placental and blood-testis barriers are usually efficient at excluding the metabolites of many chemicals, but nevertheless are refractory to others. The passage of reactive metabolites through cell membranes and across tissues can be impeded by their interactions with diverse molecular targets. Also, metabolism can alter the structure of the molecule, resulting in the modification of its ability to undergo passive transport or transport mediated by an active system. In view of their large total biomass and strategic positions at the interface between the bloodstream and the tissues, epithelia and endothelia have the potential to act as important barriers with respect to the metabolism of substances before they enter the bloodstream.

There are no barriers that can exclude all reactive metabolites or parent compounds, if the physicochemical characteristics of the chemical and its metabolites are favourable for uptake. The most relevant physicochemical parameters of a molecule in this respect are: molecular size and shape; degree of ionisation; and the relative lipid solubilities of its ionised and non-ionised forms. Other important parameters include protein binding capacity, and recognition by efflux transporters such as multidrug resistance transporters. Clearly, these properties will differ with respect to the nature of the parent compound, and for each of its metabolites. Moreover, biotransformation can generate molecules with a greatly enhanced ability to penetrate biological systems. A good example is the reductive metabolism of azo dyes that are sulphonated only on one side of the azo bridge. This results in an aromatic amine with reduced water solubility and a molecule of reduced size in comparison with the parent chemical (29).

Metabolism-mediated target organ toxicity

Differential metabolism between target organs can give rise to specific target organ toxicity (metabolism-mediated toxicity), and can be due to variations in: a) total biotransformation capacity; b)

expression levels of biotransformation enzymes and isoenzymes; and c) inducibilities of enzymes. Variations occur in the distribution of metabolising enzymes in different tissues, and also between different cells in the same organ, particularly in the case of extra-hepatic tissues such as the lung, gastro-intestinal tract, the pancreas and the brain, and especially with respect to their CYP and phase II isozyme contents (30–37).

The target organ-specific effects of some carcinogens are often attributed to differential metabolic activation. However, this might not be true for substances such as non-genotoxic carcinogens, which can exhibit high levels of tissue specificity and which often induce tumours at cytotoxic concentrations in conjunction with the induction of sustained cellular proliferation (38). In such cases, differences in receptor distribution between organs could be more important than differential biotransformation.

A good example of metabolism-mediated species-specific toxicity concerns the carcinogenicity in the lungs of mice of trichloroethylene (TRE), due to TRE activation by biotransformation enzymes in the Clara cells. However, this observation is not relevant to humans, in which there are smaller numbers of Clara cells that are not metabolically competent in this respect (39).

The role of biotransformation in determining toxicity for other endpoints is less well understood, in situations where the site and/or mechanism involved is specific to a certain target tissue (for example, neurotransmitter receptors in the brain). However, the distribution, activities and roles of CYP isoenzymes and other biotransformation enzymes in the functioning of the central and peripheral nervous systems has begun to be investigated (40). There are recent indications that CYP2D6, present in some specific brain areas and responsible for many CNS-active drug biotransformations (41–43), could be involved in the metabolism of endogenous substances in the brain (44). Also, CYP2 isoenzymes (including rat/human CYP2D1/6, CYP2E1 and CYP2B1/6) have been shown to be unevenly distributed between brain regions and brain cell types, and their regulation differs from that of the same isozymes in the liver. Therefore, while it is unlikely that CYPs in the brain contribute significantly to the overall biotransformation of xenobiotics, they might contribute to the identification of specific areas in the brain which differ in drug and metabolite levels (36). It is noteworthy that the substrates for these isoenzymes include psychotropic drugs, opioids and neurotoxins. The same reasoning could apply to other biotransformation pathways, such as glutathione-dependent activation/inactivation in the brain (45, 46).

Different tissues show a hierarchy in relation to their overall biotransformation potentials (liver >

kidney > lung > gastro-intestinal tract > nasal epithelium > placenta > brain). Nevertheless, correlation with route of exposure/administration and nature of the xenobiotic must be taken into account, in order to assess the relevance of any biotransforming hierarchy in determining organ susceptibility to toxic effects. However, such a hierarchy would only be useful in a general sense, and its relevance would very much depend on the route of administration or exposure, and the nature of the potentially toxic substance. For example, when a volatile chemical is inhaled, metabolism in the nasal epithelium and in the lung is likely to be even more important than biotransformation in the liver.

Differences in biotransformation between different organisms

Species differences

There are marked species differences in the expression and catalytic activities of the CYPs that biotransform xenobiotics. These differences can be both quantitative and qualitative, although the former are more frequently characterised by varying enzyme kinetics, which can substantially differ between rats and mice, as well as between rodents and humans. This is especially notable between the commonly-used experimental rodent models, and between these models and humans. This is a problem, particularly when assessing the toxicity of chemicals such as drugs, to which humans are intentionally exposed, so, ideally, human biotransformation should be investigated in the preclinical phase. One solution has been to develop and use transgenic mice expressing human CYPs, such as CYP2D6, and CYP3A4 (47, 48). These isozymes are expressed in amounts comparable to, or higher than, their corresponding levels in human tissues. However, a much more flexible approach is to exploit the great advantage of *in vitro* systems by varying the source of metabolism when incorporating biotransformation into *in vitro* toxicity tests, which is much more easily achieved than could ever be made possible by generating transgenic mice or by using a range of different species.

When qualitative differences in metabolism exist, there can be very far reaching consequences for obtaining correct toxicity predictions, if an inappropriate species is used (49). An example of a qualitative difference in metabolism is the lack of N-acetylation in the dog, a very important consideration when selecting a second species for determining the toxicity of aromatic amines, for instance (50). Many other examples of species-specific biotransformation were listed and discussed by Beasley (51).

All enzymes which metabolise xenobiotics display some variability (52, 53). For example, it is generally found that CYP enzyme levels in the liver display variabilities ranging from 5-fold to 100-fold. Much of this variability can be traced to genetic background, polymorphisms and single nucleotide polymorphisms (SNIPs) at the level of structural and/or regulatory sequences of xenobiotic metabolism (XME) genes. Environmental and host factors also contribute to the variability, sometimes to an even greater extent. Thus, differential distribution and the presence of the various isozymes, as well as the intracellular receptors for their induction, are mainly responsible for polymorphisms between individuals and the specific metabolic activities mentioned above (54).

Particular isozymes are associated with the metabolism and activation of specific chemical groups. For example, CYP1A1 is very active in metabolising polycyclic aromatic hydrocarbons, CYP1B1 and CYP1A2 preferentially metabolise aromatic amines, and CYP2E1 metabolises low molecular weight chemicals, whereas CYP3A4 metabolises larger molecules. CYP2D6 is especially important in human drug metabolism. However, the CYP 2C, 2B and 2D subfamilies seem to contribute very little to the bioactivation of chemicals or drugs (15).

An example often given for a CYP isozyme-specific biotransformation in toxicity, is the bioactivation by CYP2D6 of the tobacco-specific nitrosamine, NNK (55). However, this substance is also activated by other CYPs, so this is unlikely to be the basis of the frequently-quoted possible explanation for the apparent association between CYP2D6 polymorphism and lung cancer.

The existence of strain/species/gender/ethnic/age differences in susceptibility to toxicity is a well established phenomenon, and can be influenced by disease status and stress levels in the host animals (56, 57). For example, these parameters are known to affect the carcinogenicity of chemicals, due to variations in metabolic activation and detoxification, as well as differences in the formation and quenching of reactive oxygen species, and in the accumulation of parent compounds and their metabolites in tissues. Interstrain differences in biotransformation can give rise to discrepant toxicity data. Variation in the renal toxicity of chloroform is another example of the effects of such differences in biotransformation (58).

In addition, differential cytochrome expression occurs during embryonic differentiation, and this might be of importance when trying to predict reproductive toxicity *in vitro* (59, 60). Moreover, general health status can modulate the levels of CYPs, and such effects could be important when performing experiments for specific applications; an example is the effect of inflammatory mediators on P450 levels (61).

Conclusions

In conclusion, the following elements of biotransformation and biokinetics are all essential elements of toxicological evaluation, hazard identification and risk assessment: a) determining the susceptibility of a chemical to metabolism (its metabolic stability); b) identifying the principal metabolites likely to be generated; c) identifying the target organs which could be involved; d) determining other differences in metabolism (such as interspecies differences, mentioned above); e) ascertaining variations in the transport of parent chemicals and their metabolites; and f) identifying the presence of chemicals and/or their metabolites that can act as enzyme inducers or inhibitors, and their abilities to act as substrates for the different isozymes involved in biotransformation.

The Relative Importance of the Biotransformation of Pharmaceuticals and Industrial Chemicals

Safety testing in the pharmaceutical and chemical industries differs in a number of important respects. In the former, there has traditionally been a much greater emphasis on the use of rapid *in vitro* screening techniques applied in a non-regulatory context at a very early stage of drug development. This stage coincides with the availability of many closely-related candidate molecules, and is when relatively high false-positive prediction rates, and concomitant attrition rates, are not a major problem. In addition, metabolism studies are particularly important in the development of new drugs, since the vast majority of drug molecules are biotransformed. Therefore, drug metabolism is a major determinant of pharmacokinetics and the therapeutic index, and therefore particularly of the regulation of drug activity, especially in preclinical safety assessment, in which increased attention is focused on the role of new metabolites as potential mediators of the toxicity of new drug products (62).

A crucial need during drug development is to devise new molecules with the desired therapeutic activity that lack significant toxicity, and this can be particularly difficult to achieve when the same structural features of a compound are responsible for both types of biological activity (63). For these reasons, there is much impetus for the pharmaceutical industry to devise *in vitro* screening methods that take account of the need for biotransformation. The main focus of research so far has been on the involvement of the major CYP isozymes, such as CYP3A4 and CYP2D6, which show propensities for interacting with potentially toxic substrates, for inducibility, and for genetic polymorphisms. However, although other isozymes may be less-fre-

quently associated with troublesome characteristics, they are sometimes of crucial importance in the metabolism of a single specific compound.

As far as can be ascertained, no data exist on the proportion of industrial chemicals, compared with pharmaceuticals, that are biotransformed to reactive compounds. However, there is no reason to think that biotransformation is any more or any less important with respect to determining the toxicities of industrial chemicals or pharmaceuticals. Typically, but certainly not in all cases, biotransformation of pharmaceuticals leads to loss of efficacy, but is very important in determining the toxicity of drugs. It is estimated that about 20% of drugs overall are susceptible to metabolic activation, but it is difficult to be precise about this figure. Drug metabolism also contributes substantially to inter-individual differences in response, and is also often involved in drug interactions, resulting in either therapeutic failure or adverse effects (52–54). Consequently, metabolic features are among the main characteristics to be determined in a molecule that is being developed as a potential drug, particularly as many candidate drugs are capable of binding to various target proteins to form toxic adducts as a result of bioactivation (16). Therefore, the absorption, distribution, metabolism, excretion and toxicity (ADMET) properties of a candidate drug strongly influence its final clinical success (64–66).

Biotransformation and *In Vitro* Testing

One of the most frequently-cited limitations of *in vitro* tests for assessing health effects such as systemic toxicity, reproductive toxicity and carcinogenicity, is qualitative and quantitative deficiencies in the biotransformation of test chemicals, in comparison with the *in vivo* situation, i.e. the lack of a specific metabolic component. Despite this, apart from genotoxicity testing and hepatotoxicity studies (usually by default when primary hepatocytes are used), there has been relatively little consideration of the potential importance of biotransformation as a component of the *in vitro* toxicity testing methods that have so far been developed and validated for regulatory use (67, 68).

When no biotransformation has been specifically incorporated into an *in vitro* test, any good predictivity of such a method could have been due to one or more of the following: a) the toxicity endpoint being predicted has been induced by the parent substance without the need for the generation of reactive metabolites; b) the residual biotransformation capacity of the indicator cells has been sufficient; and c) biotransformation was not crucial for inducing the endpoint.

However, when using *in vitro* methods to assess health effects, such as endocrine disruption, acute toxicity, neurotoxicity, embryotoxicity, skin sensi-

sation and chronic toxicity, including carcinogenicity and hepatotoxicity in particular (69–73; Prieto, in preparation), there is an important need to take account of the contribution of metabolism to toxicity. The importance of using metabolising systems in *in vitro* toxicity tests has been considered in some detail by Zucco *et al.* (74). In the case of sensitisation, it is well-known that allergens can arise from parent molecules (prohaptens) following their metabolism to electrophiles that can covalently bind to immunologically active proteins in the skin. Such reactions are often catalysed by cytochrome P450 enzymes in this organ, as well as by monoamine oxidases and peroxidases following oxidative stress induced by the presence of a xenobiotic.

A further example is the food flavouring substance, 5-hydroxymethyl furfural, which can be bioactivated to 5-[(sulphoxy)methyl] furfural, through sulphonation of its allylic hydroxyl functional group, catalysed by SULT. The resulting ester has been demonstrated to induce genotoxic effects, but only when the appropriate cofactor (3-phosphoadenosine-5-phosphosulphate; PAPS) is added to the metabolic activation system in the Ames test (75).

The advantages of incorporating biotransformation into *in vitro* test systems

The ability to add metabolism to *in vitro* assays, unlike the situation with animals, has the additional benefit that it is possible to use human sources of metabolism and to model individual differences in metabolism (pharmacogenetics) *in vitro* (76–78). An important principle concerning the use of *in vitro* systems that has clearly emerged over the last 10 years, is that it is crucial to characterise the functionality of both primary models and cell lines before they are used for toxicity studies, because it cannot be assumed that tissue-specific functions are maintained *in vitro* (79).

Problems with adding metabolism to *in vitro* test systems

Several major problems are encountered in studying metabolism-related toxicity *in vitro*: a) modelling human metabolism (80); b) maintaining tissue-specific functions *in vitro*; c) selecting an appropriate xenobiotic metabolising system; d) keeping enzyme activity stable over time; and e) the adverse effects to toxicity-indicator cells of subcellular metabolising fractions. The third problem (c) is crucial for improving target organ-specific *in vitro* models, as is discussed below. It is due mainly to the complexity of these issues that the development of relevant metabolic competents for *in vitro* tests for assessing different human health effects has made relatively little progress.

Two further problems with respect to modelling biotransformation *in vitro* concern: a) the testing of mixtures of chemicals that might require different enzyme systems (81); and b) the inactivation of exogenous biotransformation systems, due to exposure to certain solvents and test substances (82).

Existing *In Vitro* Systems with Metabolic Competence

Introduction

There are two main ways to take account of biotransformation when using *in vitro* systems for toxicity testing: a) by testing the known principal and important metabolites of the parent substance of interest; and b) by providing indicator cells with metabolic competence, by adding metabolising systems either endogenously or exogenously. Currently, the first strategy is much more rarely adopted than the latter.

Testing metabolites

If metabolites can be identified, isolated and synthesised they can be tested *in vitro*, as was recently done by Freyberger and Sholz (83) in their studies on the endocrine disrupting potentials of several chemicals. Examples of other studies in which metabolites have been tested in order to study their toxicity can be found in references 62 and 84.

However, such work depends on having knowledge of the likely metabolites of the substance of interest, and also having samples of each of its main metabolites. In addition, in some cases, the most dangerous metabolites are the more reactive and more unstable ones. Thus, this approach is severely limited, although it is a way of avoiding some potential technical problems.

Identifying metabolites is time-consuming, since it requires the use of radio-labelled chemicals and/or sophisticated analytical techniques, such as high field nuclear magnetic resonance (NMR), high performance liquid chromatography (HPLC) coupled with mass spectrometry (LC/MS) and/or NMR, tandem MS, hydrogen-deuterium exchange and derivatisation methods and antibody binding (85–87). However, recent advances in time-of-flight (TOF)-MS have made it feasible to tentatively identify principal metabolites and to rapidly construct a metabolic route map (88). This tentative map can then be refined and modified by using the above-mentioned, more definitive techniques. Since metabolites can be tentatively identified quickly on the basis of *in vitro* studies, it has also become feasible to use more-comprehensive enzyme systems in metabolite identification studies.

Adding a metabolising system to indicator cells

This is by far the most-commonly adopted strategy for taking account of the role of biotransformation in toxicity testing. The metabolising systems used can be either subcellular or cellular (Table 3).

Subcellular metabolising systems

Subcellular systems are added to *in vitro* test methods as enzyme homogenates. Such homogenates can comprise: a) purified enzymes; b) cytosolic soluble enzyme fractions (S100 supernatant); c) microsomal particulate enzyme fractions (S100 pellet); and d) post-mitochondrial S9 supernatant (consisting of both cytosol and microsomes). All of these systems are capable of activating and/or detoxifying xenobiotics to differing extents and with varying results, depending on the chemical being studied and the co-factors added. The latter can be taken from commercially-available frozen stocks for which quality controls have been established.

The above systems have been used mainly in genotoxicity assays for regulatory applications. For routine genotoxicity screening, male rat induced-liver S9 is the first choice for an exogenous metabolising system, either from animals induced with aroclor, a mixture of phenobarbitone and benzo-naphthoflavone, or a variety of other enzyme inducers (89–91). Table 4 shows examples of the OECD test guidelines in which S9 is incorporated as a metabolically competent source. The *Salmonella* mutagenicity assay was one of the first tests that involved the routine use of a metabolically competent source for regulatory application. A search on 3745 dossiers in the European Chemicals Bureau new chemicals database shows clearly that 30% of chemicals which are positive in the Ames test required the use of S9 (Table 5). However, it is acknowledged that S9 is not an optimal source of biotransformation enzymes, since it represents an extracellular source of metabolism; also, it does not accurately reflect the capacity of human cells for biotransformation, since it is usually obtained from induced rat liver.

One approach to refining a genotoxicity assay has been to change the metabolising system with regard

Table 3: Comparison of *in vitro* enzyme sources used in preclinical research

Enzyme source	Availability	Advantages	Disadvantages
Microsomes	Relatively good; from transplantation or commercial sources	Relatively inexpensive; easy storage	Contain only phase I enzymes and UDP-glucuronosyl transferases (cofactor and, in the latter case; detergent/alamethicin addition is necessary; require strictly specific substrates and inhibitors or antibodies for individual enzymes)
cDNA-expressed individual CYP forms (or multiple CYPs)	Good, commercially available	Can be used with HTS substrates; the role of individual CYPs in the metabolism of an NCE can easily be studied	The effects of only one or a few enzymes at a time can be evaluated
Immortalised cell lines	Available on request, not many adequately characterised cell lines exist	Unlimited source of enzymes; good cryopreservability	The expression of most enzymes is poor or absent, if characterised at all; genotypic and phenotypic instability
Primary hepatocyte cultures	Relatively difficult to obtain, fresh tissue needed; commercially available; cryopreservation possible (but more difficult than with cell lines)	Contain the whole complement; the induction effect of an NCE can be studied; active transport systems are present	Require specific techniques and well-established procedures; the levels of many enzymes decrease during culture
Liver slices	Relatively difficult to obtain, fresh tissue mainly used; cryopreservation possible	Contain the whole complement of DMEs and cell-cell connections; the induction effect of a xenobiotic can be studied	Require specific techniques and well-established procedures; limited viability (< 24 hours), damaged cells at surface. marginal aeration of inner cells, slow transport through tissue

HTS = high throughput screening; NCE = new chemical entity.

Table 4: EC and OECD Test Guidelines for genotoxicity that require the addition of an exogenous metabolising system

EC	OECD	Test
B13–14	471	Bacterial reverse mutation test
B10	473	Mammalian chromosome aberration test
B17	476	Mammalian cell gene mutation test
B19	479	Sister chromatid exchange assay in mammalian cells (SCE)
B15	480	<i>Saccharomyces cerevisiae</i> gene mutation assay
B16	481	<i>Saccharomyces cerevisiae</i> mitotic recombination assay
B18	482	Unscheduled DNA synthesis (USD) in mammalian cells

to the type of system, organ, sex, species, and, in particular, to permit the use of human-derived sources of enzymes (29). In the case of kidney-specific toxicity of TRE discussed earlier, the bacterial mutagenicity of this chemical was only apparent when microsomes from the kidney were used (58).

Cellular metabolising systems

Cellular enzyme systems can also be used in *in vitro* assays (92), and these fall into one of three main categories: a) residual metabolism of the indicator cells; b) the use of co-culture systems comprising the indicator cells and a metabolically-competent cell source (for example, freshly isolated hepatocytes or genetically-engineered cell lines); and c) the use of a cell-based system that simultaneously acts as both an indicator of toxicity and a source of metabolism.

The currently available and most promising metabolic competent cell sources comprise a diversity of systems, ranging from subcellular fractions, precision-cut tissue slices, hepatocyte suspensions, long-term hepatocyte cultures (93) and stem cells,

to various cell lines, such as Hep G2 and Hepa RG (94), and cells expressing various cDNAs for biotransformation enzymes.

Residual metabolism in indicator cells

The contribution of the indicator cells themselves to metabolism, with or without the addition of exogenous metabolism, can be a problem (which is unavoidable unless specific inhibitors are used). Although such residual metabolism is usually low, particularly with respect to phase I enzymes, it should not be overlooked when interpreting toxicity data (29). Often, the level and specificity of any such residual metabolism are unknown, although it is usually low, and this can complicate the situation (as will be discussed later, in more detail). However, the importance of routinely characterising any inherent residual biotransformation competence in any *in vitro* test, before it is developed and recommended for detecting toxicity, was recognised. This should include characterisation of phase I and II enzyme activities, and the levels of other relevant cellular constituents and activities, such as glutathione, per-

Table 5: Numbers of chemicals in the ECB new chemicals database that were positive in the *Salmonella* mutagenicity assay with respect to the use of S9 mix

Result with S9	Result without S9	Number of chemicals	% positive
+	+	264	62
+	-	128	30
-	+	33	8
Total number of positive chemicals	425 ^a		
Total number of negative chemicals	3320 ^b		
Total number of chemicals	3745		

^a11% of the total number of chemicals were positive with and without S9; ^b89% of the total number of chemicals were negative with or without S9.

oxidase and catalase levels, as undertaken by a number of authors (29, 95–101; Table 6). Moreover, the metabolic competence of indicator cells should be checked periodically, to ensure that basal activities have not altered significantly.

The use of co-culture systems

It is important to note that co-cultures can be established by using both cell types from primary cultures, or from cell lines, or by mixing a primary cell

culture with a cell line. Several physiologically relevant models have been established for liver cells, including co-cultures, by using primary cells attached to an extracellular matrix, such as collagen, as in sandwich cultures (102, 103), or by using liver spheroids. The immobilisation of hepatocytes on alginate gel (Liverbeads™) or alginate biospheres has been shown to be a useful technique for studying the biotransforming capacity of hepatocytes (104, 105). Choi *et al.* (106) described a double-layered co-culture system, comprising two cell lines, Hep G2 and Caco-2, which showed a reduced

Table 6: The inherent xenobiotic metabolising potential of some commonly-used indicator cell lines

Cell type	CYP	Phase II enzymes	Other enzymes	Capacity for activation/detoxification
Chinese hamster ovary (CHO)	None	GST	Catalase, superoxide dismutase, peroxidase; nitroreductase high	Low/?
Chinese hamster lung V79 fibroblasts	None	GST	Catalase, superoxide dismutase, peroxidase; nitroreductase low	Low/?
Human lymphoblast TK6 cells	None	GST	Catalase, superoxide dismutase, peroxidase	Low/low
Mouse lymphoma L5178Y cells	None	GST	Catalase, superoxide dismutase, peroxidase; nitroreductase low	Low/?
Mouse C3H10T1/2 cells	Some evidence for 2A1 and 1A2 ^a	GST	Catalase, superoxide dismutase, peroxidase	Medium/?
Primary rat hepatocytes	Various	GST	Catalase, superoxide dismutase, peroxidase isozymes	High/medium
Hep G2 cells	Various isozymes present but not necessarily expressed	?	Probably	Medium/?
Human hepatoma BC2	Various isozymes present and expressed	GST, UGT	?	Responded to inducers; activation likely
Human pulmonary A549	Some CYP activity but different to Hep G2	?	?	Yes, shown for B[a]P
LLC-PK1	Very low activities ^a	?	?	?
HepaRG (patented by INSERM)	A variety of phase I reactions demonstrated	GST	?	CYPs 1A2 and 3A4 have been shown to be inducible

^asee also Gonzalez & Tarloff (194).

level of toxicity of benzo[a]pyrene, compared with the use of mono-cultures. This result agreed with *in vivo* oral acute toxicity data.

All of these sophisticated culture models show partially improved characteristics over regular hepatocyte monolayer cultures, whether comprised of primary cells or cell lines. A further example is the use of primary cultures of rat hepatocytes to generate metabolites, the neurotoxicities of which were assayed in embryonic chicken brain primary cell cultures, as toxicity targets in the same co-culture system (107). Also, Coecke *et al.* (108) have used genetically-engineered cell lines with neural cells to model metabolism-mediated neurotoxicity. Lastly, Bremer *et al.* (109) used the co-culture of embryonic stem cells and metabolically competent cells to demonstrate the embryotoxicity of cyclophosphamide, a chemical known to require metabolic activation to exert its genotoxic and teratogenic activities.

At present, however, no approach has produced the desired total profile of maintained metabolism/tissue-specific functions. Also, none of these sophisticated culture models are used regularly in toxicology studies, partly because they seem to be less sensitive than conventional hepatocyte monolayer cultures, and because the standardised procedures have not been established (110).

This is a potential way to overcome situations where it is impossible to use a co-culture method due to incompatibility between the two cell types involved. For example, such problems could arise because of the impossibility of finding a suitable medium that will support both component cell types. In such cases, it might be possible in certain circumstances to expose the indicator cells to metabolites in pre-conditioned medium, free of the cells that produced them. However, this method suffers from the serious drawback that short-lived metabolites might go undetected.

The use of precision-cut tissue slices in culture from various organs is another technique which is widely-used in the pharmaceutical industry (111). Martignoni *et al.* (112) have demonstrated that cryopreserved liver slices from a number of different species retained a series of marker enzymes for phase I and II metabolism. However, the relatively short life-span of tissue slices limits their applicability to many situations in toxicity assessment (113). Nevertheless, it might be possible to use slices for the rapid detection of toxicity in integrated reporter gene assays and in genomic and proteomic studies (114). For kidney cell culture, the use of membranous culture inserts has led to more-physiological conditions (115).

The use of genetically engineered cell lines

Many different cell lines have been genetically engineered to express various phase I and phase II

enzymes, or a combination of both types, for use in toxicity testing (Table 2; 77, 116–126). Genetically engineered cell lines expressing various CYPs have been produced from mammalian cell lines such as V79, CHO-AS52, CHL, C3H10t1/2, NIH-3T3, PC-12, COS, AHH-1, AHH-1 TK[±], MCL-5, THLE, BEAS-2B, Hep G2, NHBE (127, 128).

Some of these cell lines have a number of benefits, including a stable diploid karyotype and no CYP background activity, and they can be transfected with rodent and human CYPs. This permits an investigation of the contributions of specific isozymes to metabolism, as well as studies on species specificity. The initial approach was to transfect stable cell lines with individual CYPs or other metabolising systems, genes, or combinations of them. Such models can be used to answer specific mechanistic questions, but have so far not been widely used for toxicity studies (127, 129). It has been recognised that, without the full complement of metabolic functions, these models lead to the generation of data which has to be interpreted with caution. Also, the inducibility of the CYP system *in vivo* is not reproduced fully in these *in vitro* models. Another approach in this area has been to transfect cells with various transcription factors, in the hope that this will lead to a cascade effect that could up-regulate a large number of metabolism-related genes (130). However, so far, only a limited success has been reported with this approach.

The advantages and limitations of the different systems

Exogenous versus endogenous biotransformation

The main advantage of using exogenous biotransformation systems is that the test compound does not have to enter the cells before being metabolised, because it is in direct contact with the enzymes. However, unless the toxicity detection system is subcellular (for example, a receptor binding assay), the resulting metabolites have to enter the cells to exert their toxicity, a process that can be exacerbated if they are rendered more polar, and therefore less bioavailable, by phase I metabolism. In the case of co-culture systems, xenobiotics have to enter the metabolically-competent cells, and then exit them to subsequently enter the indicator cells (131). However, in cells genetically engineered both to metabolise xenobiotics and to detect toxicity, metabolites are generated in close proximity to the target site for toxicity. Thus, intracellular activation is more likely to lead to toxicity.

Metabolites that cause toxicity by interacting with intracellular or membrane receptors at sites remote from their formation (e.g. in the liver), will reach their target sites via the bloodstream. In

such cases, biotransformation by adding enzymes exogenously, or by using a co-culture system (for example, indicator cells with hepatocytes), might constitute a more realistic approach to assessing toxicity *in vitro*. On the contrary, where reactive metabolites are formed that cause toxicity common to all cell types (such as DNA damage), the use of target cells that have their own biotransformation capacity (either intrinsic or introduced by genetic modification) is likely to be the best system of choice.

The need for in vitro biokinetics

It should be remembered that the addition of metabolic competence to a cell system, especially if exogenous metabolising systems are used, might affect the bioavailability of the chemical at the target site.

Discrepant results *in vitro* can arise from inherent inadequacies of the test system or from artefactual effects — for example, where a reactive, potentially toxic metabolite is quenched by an intracellular or extracellular component, as discussed above, or where a metabolite or test material cannot enter the target indicator cells. It is therefore important to assess the bioavailability, biokinetics and biodynamics of substances tested in *in vitro* assays. Glden and Seibert (132) have shown that *in vitro* data can be corrected to account for compound bioavailability in *in vitro* test systems. However, more information is required on how *in vitro* biokinetics and biodynamics should be quantified, expressed and applied, and this should be the subject of a future ECVAM workshop. Figure 1 summarises the main factors involved.

Further considerations

Further considerations to be taken into account when choosing a metabolising system, include: a) the need to model *in vitro* entero-hepatic recirculation, which involves exogenous activation by gut epithelia and bacteria; b) the fact that primary cells and cell lines might be differentially susceptible to the same chemical, due to differences other than metabolic competence — for example, the presence of different receptors and transporters, and differences in cytology; and c) the well-known problem of preferential activation *in vitro*. Preferential activation occurs because 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 co-factors for phase II reactions, but is more usually accounted for by using whole cells such as hepatocytes (133) and/or other cells that express both phase I and II reactions.

Conclusions

It was agreed that the various methods available for taking account of biotransformation have benefits and disadvantages, and that no single method is ideal. For example, there are still problems with the limited number of different metabolising enzymes that can be transfected into cell lines, and because low levels of expression are often obtained. Both of these problems need to be overcome, in order to increase the usefulness of genetically engineered cell lines.

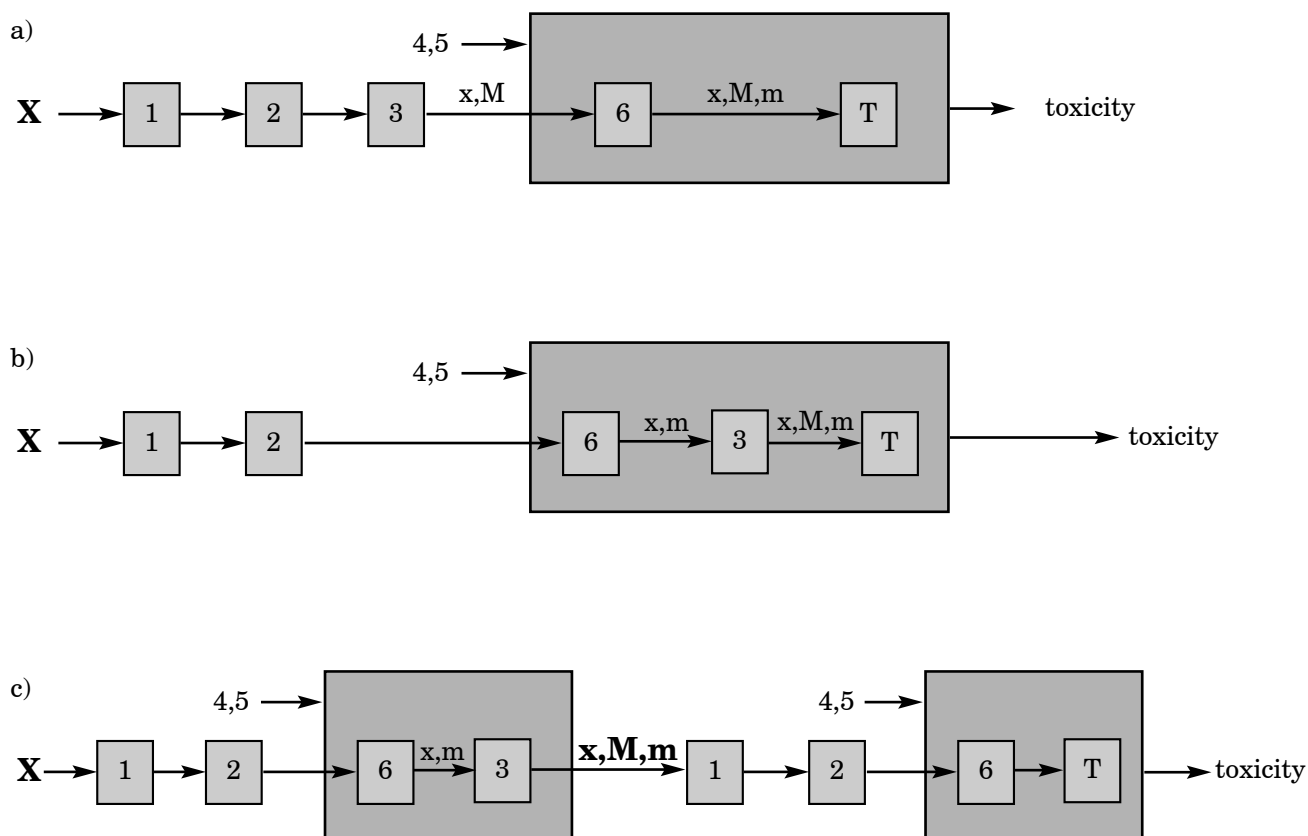
To answer a specific question, the most appropriate system should be selected, and in particular, to permit the best extrapolation of the *in vitro* data produced to the *in vivo* situation. The work of Boess *et al.* (134) is noteworthy, since they have used a genomic approach to show that rat liver slices possessed the strongest similarity to liver tissue with regard to mRNA expression, in comparison with the expression profiles of two rat cell lines and primary hepatocytes.

Existing Methods to Introduce Metabolic Competence in *In Vitro* Toxicity Testing

Introduction

Biotransformation is chemical-specific, although some useful, although probably not perfect, rules can be developed for single chemical classes. It is a common rule that each chemical has its own routes and rates of metabolism, due to specific affinities to various enzymes and turnover rates (dictated by specific structural complementarities toward various enzymes). In order to accurately predict the metabolic fate and rate of biotransformation of any chemical, a screening method for metabolism should be relatively comprehensive, i.e. it should cover the major parts of the xenobiotic-metabolising machinery.

Metabolism screening is conducted to identify: a) whether a chemical is likely to be metabolically activated or detoxified; b) the major, and all other reasonable, phase I and II metabolites, as well as the ones likely to be toxic, under specific sets of conditions of metabolism and toxicity endpoint; c) the principal pathways for activation and detoxification, preferably organised in the form of a metabolic tree for each chemical; d) the major CYP isozymes involved (to give an indication of the risk to a particular species, including humans); e) the isozymes of other important biotransformation enzymes, such as NAT and SULT; and f) the importance of using a metabolising system in *in vitro* efficacy and toxicity tests. Screening is also useful for selecting an appropriate non-rodent second species, and to prioritise chemicals for testing in a non-regulatory context.

Figure 1: *In vitro* biokinetics and metabolising systems

a = exogenous metabolising system; b = endogenous metabolising system; c = co-culture metabolising system. X = parent chemical; M = metabolites from various sources (upper/lower case depicts relative amounts); 1, 2 = media components (e.g. serum, other proteins); 3 = added metabolism (exogenous or endogenous); 4 = cytoplasmic membrane; 5 = transporters/surface receptors; 6 = intracellular residual metabolism; T = target for toxicity. Physical properties of X and M (e.g. molecular size, shape, lipophilicity, ionisability) determine bioavailability to the various cellular systems for metabolism/toxicity.

Many methods (see Table 7) can be used to screen chemicals for their susceptibility to metabolism, and to establish the role of metabolism in their toxicity. The source of metabolism can also be varied according to sex, species and tissue, as well as enzyme, or by the use of enzyme inducers or inhibitors specific for particular isozymes (see below). These systems are also used in conjunction with a number of extensive databases on metabolism, such as the MDL metabolite database, and extensive compendia on biochemical transformation (see www.mdli.com/products/predictive/metabolite/index.jsp).

The use of simple cell-based screens

Determining whether a chemical is likely to be activated or detoxified is complicated by the fact

that it is necessary to assess the role of metabolism for all the toxicity endpoints of interest, since the same chemical might be toxic for a particular endpoint without needing to be metabolised, as well as being activated or detoxified for another endpoint. However, a simple method for assessing the role of metabolism in the toxicity of a chemical would be to assess its basal cytotoxicity with and without metabolism, either by using metabolically competent and non-competent indicator cells, or by using the same cell in the presence of a metabolic inducer or inhibitor, or with and without the addition of a metabolising system. This can be conveniently done in industry by performing the *Salmonella* mutagenicity assay with and without liver S9, since this test involves genotoxicity, a key toxicity endpoint in the development of chemicals and drugs. However, the results of such a test will

Table 7: Systems for biotransformation screening

In silico prediction systems (see Table 9)

Pure enzymes

Enzyme fractions

Post-mitochondrial supernatant (S9)

Microsomes

Cytosol

Tissue slices

Gut microflora metabolism

Prostaglandin H synthase system (see Table 1)

Use of specific enzyme inhibitors

Use of specific co-factors for phase I and phase II (conjugating) enzymes

Metabolically competent cells (e.g. hepatocytes) from different species

Genetically engineered cell lines expressing single or multiple CYPs and phase II enzymes

Co-cultures of metabolising cells and toxicity indicator cells

Use of metabolising cells as toxicity indicator cells

Host-mediated assays

Metabolite identification and testing

Testing of known or suspected metabolites

not necessarily be relevant for predicting the importance of metabolism for other forms of toxicity for the same chemical.

Yueh *et al.* (135) have described assays involving the use of the Hep G2 cell line for detecting chemicals that either induce CYP1A by binding to the Ah receptor linked to a promoter region for the enzyme, luciferase, in a reporter gene, or that inhibit the activity of the enzyme. The authors suggest that this assay could be adapted for high throughput screening, so that a requirement for metabolism could be rapidly identified.

Another simple way to assess the susceptibility of a chemical to metabolism (or, more accurately, the formation of an electrophilic reactive intermediate) is to measure its binding to protein in the presence and absence of a source of metabolism. Metabolic pathways can also be monitored by exposing cells to radioactively labelled drugs.

The use of microorganisms

There has been much interest in the use of microorganisms, particularly certain fungi, as surrogates for mammalian metabolism, to investigate the likely metabolites produced from a wide range of xenobiotics (86, 136, 137). The principal assumption made is that fungi, being eukaryotic, share metabolic biotransformation pathways similar to those in higher eukaryotes, including mammals, and this has been verified for a variety of different substrates. Indeed, both phase I and II enzyme activities have been demonstrated in organisms such as *Cunninghamella echinulata*,

Saccharomyces cerevisiae, and species of the genus, *Aspergillus*. However, there are several cases where the expected mammalian metabolites are not generated, or where metabolites are formed which are not formed in mammals. It is well-known, for example, that the use of *S. cerevisiae* in genotoxicity studies, with and without an added mammalian metabolism system, and without optimising the culture conditions for maximum residual biotransformation activity, can yield different results from those obtained with other test systems incorporating a mammalian biotransformation system (138). However, yeast cells, genetically engineered to express human CYPs, have proved useful in drug metabolism studies (139). It is therefore suggested that the use of fungi for metabolism screening should be approached with caution, particularly since many mammalian-based methods are now widely available and can be easier to use.

The use of test batteries of cells

By using immortalised cells, or established cell lines genetically engineered to express various phase I and II enzymes, it is possible for one laboratory to screen more than 200 chemicals per month (Johannes Doehmer, personal communication, see www.genpharmtox.com/services/Metabolism). It is therefore recommended that the use of batteries of cell lines with different and specific metabolising capacities for screening large numbers of chemicals for their susceptibility to metabolism, should be actively developed and promoted, especially as part of an integrated testing strategy for assessing the

toxicity of existing chemicals in compliance with the EU REACH System (140).

The use of *in silico* methods

Overview

There are three main ways of predicting metabolism by using computerised methods: a) modelling of whole molecules and the three-dimensional interactions between chemical substrates and biotransformation enzymes (3D-QSAR); b) (Q)SAR studies on the binding of chemicals with the active sites of these enzymes; and c) expert systems, that combine several of these approaches (for reviews, see 141–144). The principles of these various physico-chemical methods are summarised in Tables 8 and 9. They primarily involve calculating the quantum mechanical properties of molecules in order to establish their intrinsic reactivities, then predicting the interactions between metabolising enzymes and other biological receptors, based on electronic and 3D considerations. This information is then integrated, in order to predict susceptibility to metabolism, both qualitatively and quantitatively.

Molecular and QSAR modelling

Such metabolism modelling and (Q)SAR studies have been greatly facilitated by the availability of

the crystal structures of some biotransformation enzymes, especially the CYPs. The majority of CYPs for which such structures are available (thus far, 21 in total) are microbial in origin, although the latest available information indicates that there are crystal structures for three human CYPs (2C8, 2C9 and 3A4), and one rabbit CYP (2C5/3) (145). Vedani (146) has recently described the use of the crystal structure of human CYP3A4 to develop a computational model to accurately predict the docking potential of a number of diverse putative ligands (substrates for the isozyme). A further useful resource for modelling metabolism is the availability of information on a wide range of protein structures (see www.rcsb.org.pdb). The structures and mechanisms of substrate binding of various other CYPs and other enzymes, such as epoxide hydrolase, have been developed by homology modelling (147–149).

Lewis (150) has recently published the results of a large study, involving the development of QSAR models for a range of substrates for the human CYP2 family of enzymes. He reported good correlations between binding affinities to, and molecular interactions with, the active sites of the various isozymes and a number of physicochemical parameters of the substrates. The model permitted the estimation of substrate binding energy for a given isozyme from a combination of energy terms, including hydrogen bonding, desolvation, bond energy changes and an electronic parameter.

Table 8: Molecular approaches to analysing xenobiotic metabolism

Method	Principle
Determining intrinsic reactivity of a substrate	Determination of electronic charge distribution, E_{lumo} , E_{homo} molecular orbital (MO) energies and related molecular parameters
Predicting rates of specific enzyme reactions	Quantum mechanical and thermodynamic calculations of reactivity in relation to relative transition energies of reactions
Molecular modelling of enzymes	Use of crystal structures (of available microbial CYPs) to model active sites followed by docking experiments with potential substrates
Pharmacophore modelling	Determination of structural requirements for substrate binding to active sites
Molecular field analysis	CoMFA as applied to congeneric series of enzyme substrates and inhibitors to generate QSARs based on 3D properties correlated with K_m
Modelling specific enzyme-mediated biotransformation reactions and substrate requirements	Developing SARs for interactions between specific enzymes and potential substrates
Application of QSARs without molecular modelling	Using physical parameters (e.g. log octanol/water coefficient) to predict affinity constants for substrates and inhibitors

The use of expert systems

Expert systems can be used either specifically or coupled with rule bases for predicting general toxicity (151, 152). Examples of this include CASE and METACASE, HazardExpert and MetabolExpert, Derek for Windows and Meteor, as well as the TIMES metabolism simulator software (141, 153, 154; Table 9). In this way, it is possible to identify predicted metabolites in one program, and process them for structural alerts by using the other related program.

Such programs can also be used to determine the log P values of metabolites and parent compounds, and to predict important properties of compounds, such as skin permeability. In addition, Meteor (141, 153), provides information on metabolites, metabolic trees, and metabolic pathways.

One of the problems of metabolism prediction programs is that they are capable of generating very large numbers of metabolites from one parent molecule, without necessarily giving any indication of whether these are likely to be major or minor metabolites under specific conditions. However, some models do provide such information (155), while others can be controlled to limit the numbers formed, or to set the species, target organ, sex and other parameters, such as whether only phase I or II reactions, or both, are involved.

Meteor controls the number of metabolites generated at any one time from a query molecule, by using absolute and relative reasoning modelling techniques that are able to prioritise biotransformations (156). Reasoning requires the combined use of these two techniques to cope with the complexities of different biochemical pathways that might be competing for the same substrate. Meteor is also supplied with a knowledge base editor, so that users can incorporate their own sets of biotransformation reactions and rules into the system.

Like Meteor, TIMES involves the use of a heuristic algorithm to produce plausible biotransformation pathways from a query molecule, by using rules developed from a comprehensive library of biotransformations (153, 157). The generation of metabolites by TIMES can also be limited to the most likely ones or extended to include less likely ones. The developers of the software have also integrated reactivity models for various macromolecular interactions (for example, for mutagenicity and sensitisation), to enable the software to simulate the generation of reactive metabolites by specific metabolising systems, such as S9.

COMPACT is a different system, which analyses the ability of a molecule to fit into the active site of the CYP1A1 isozyme (and some other CYP isozymes), by modelling molecular shape (planarity or area/depth) and chemical reactivity (covalent bond formation). The use of COMPACT is therefore

limited to molecules that are activated by these CYP isozymes (151).

Although *in silico* metabolism prediction systems may have great potential, they all require further improvement before they can be considered acceptable for specific applications (158). Moreover, like all *in silico* prediction systems, they need to be validated, and this is proving controversial (159). No screening system can be totally comprehensive, so the coverage of any one system is a compromise between what is ideal and what is feasible.

The computational modelling of metabolic reactions is a highly complex task, and more use should be made of available mathematical simulation software, such as DBsolve (160), to facilitate the process.

Biotransformation and ADME

Introduction

The prediction of ADME (absorption, distribution, metabolism and excretion) is an integral part of drug development and assessment of the safety of these and other chemicals. It is traditionally undertaken by using whole animal methods, in conjunction with kinetic studies, by using metabolism cages, and according to standard test guidelines and protocols. However, the use of computational methods to model biokinetics is increasingly being adopted, particularly in the pharmaceutical industry, as part of high throughput screening strategies (Table 10).

Biokinetic modelling

The use of biokinetic (or physiologically-based pharmacokinetic — PBPK) modelling outside the pharmaceutical industry is slowly beginning to increase (161, 162). This approach can provide information that is particularly useful in the design of *in vivo* toxicokinetic studies, and for interpreting *in vitro* and *in vivo* data. Biokinetic modelling is also useful in addressing the fundamental problem in risk assessment of the need to relate the effects detected at the dose level applied to the test system (the external dose) with the effects that might be caused by the dose that actually reaches the target in the whole animal (the internal dose). The internal target organ dose can be predicted by undertaking toxicokinetic studies, taking account of ADME. A better, biologically-based, dose-response model of *in vivo* toxicity can then be developed from external dose data. Thus, the information from biokinetic modelling is very useful for identifying concentrations likely to be present at target sites *in vivo* following the dosing of animals, so that objective

Table 9: Expert systems, QSARS and software for predicting toxicity and metabolism

Name	Supplier	Website	Endpoints predicted
CASE/MCASE/ CASETOX; METACASE	MultiCASE Inc	www.multicase.com	Carcinogenicity, mutagenicity, teratogenicity, mammalian acute and chronic toxicities and others; phase I and II metabolism
Derek for Windows; Meteor	LHASA Ltd	www.lhasalimited.org	Carcinogenicity, genotoxicity, skin sensitisation, irritation, respiratory sensitisation and others; phase I and II metabolism
Hazard Expert; Metabol Expert	CompuDrug Ltd	www.compudrug.com	Oncogenicity, mutagenicity, teratogenicity, membrane irritation, sensitivity, immunotoxicity, neurotoxicity, bioavailability and bioaccumulation; phase I and II metabolism
TOPKAT	Accelrys Inc	www.accelrys.com/ products/topkat	Carcinogenicity, mutagenicity, developmental toxicity, skin sensitisation, eye irritancy, biodegradability and various mammalian acute and chronic toxicities
OncoLogic	LogiChem Inc	www.logichem.com	Carcinogenicity
COMPACT	Prof David Lewis, University of Surrey	www.surrey.ac.uk/ SBMS	Carcinogenicity via metabolic activation by CYP1A and CYP2E subfamilies
PASS	Laboratory of structure function based drug design, V.N. Orechovich Institute of Biomedical Chemistry	www.ibmh.msk.su/ PASS/	Mutagenicity, carcinogenicity, teratogenicity, embryotoxicity and many other effects
EPI Suite of Programs	Syracuse Research Corporation	www.syrres.com/esc/ est_soft.htm	Biodegradation (BioWin), dermal permeability (DermWin) and other environmental effects
ToxScope™	LeadScope Inc	www.leadscope.com/ products/txs.htm	Acute toxicity, hepatotoxicity, mutagenicity, carcinogenicity
Tox Filter	Pharma Algorithms	www.ap-algorithms. com/tox_filter.htm	Mammalian acute toxicities
OASIS Software Packages	Laboratory of Mathematical Chemistry, Bourgas University	http://omega.btu.bg/ software.php	Biodegradation, acute toxicity, phototoxicity, mutagenicity, ER/AR binding affinity (CATABOL), skin sensitisation, mutagenicity (TIMES)
Metasite	Molecular Discovery Ltd	www.moldiscovery. com	Phase I and II metabolism
BRENDA	University of Koeln	www.brenda.uni-koeln. de	Enzyme data and metabolic information
METALOG Database	Imperial College London	www.beaconprojects. org.uk/metalog.htm	Metabolic pathway prediction

Table 10: Biokinetic PBPK and ADME prediction programs

Name	Supplier	Website	Properties predicted
OraSpotter™	ZYX Bio	www.zyxbio.com	Absorption in GI tract
iDEA pkEXPRESS™	LION bioscience	www.lionbioscience.com	Absorption in GI tract; systemic circulation or bioavailability; plasma concentration; elimination
Megen100	Health & Safety Lab	www.hsl.gov.uk/capabilities/pbpbk.htm	Oral and i.v. absorption; concentration/time profiles for plasma and major organs and tissues; liver metabolism
CSPredict	ChemSilico	www.chemsilico.com	Oral absorption
PK-Sim®	Bayer Technology Services	www.bayertechnology.com	Oral absorption; concentration/time profiles for plasma and major organs; bioavailability; renal and biliary excretion
QikProp	Schrodinger	www.schrodinger.com	Free energies of solvation (hexadecane, octanol, water); BBB, Caco-2, MDCK, skin permeabilities
GastroPlus™	Simulations Plus Inc	www.simulations-plus.com	Absorption in GI tract; concentration/time profiles for plasma; liver metabolism
Cloe PK®	Cyprotex	www.cyprotex.com	Potential exposure; absorption in GI tract; plasma, tissue and organ concentrations; renal excretion; hepatic metabolism
COSMOfrag	COSMO	andreas.klamt@cosmologic.de	Drugs and pesticides; ADME high throughput screening based on quantum chemistry

concentrations can be applied to *in vitro* test systems.

PBPK modelling involves combining experimental results, *in vitro* data, data from the literature, and computational techniques, and extrapolating data from *in vitro* studies between species (163). Organs or groups of organs are considered as discrete interconnected compartments with physiological volumes and blood flows. These models can account for physiological influences and can be used to estimate internal tissue dose, permitting extrapolation between species, doses and routes. PBPK models simulate kinetic profiles from exposure data and a number of physiological and chemical-specific parameters generated outside the model. The physiological parameters include, in particular, species-dependent tissue volumes and blood flows. Chemical-specific parameters, and often species-dependent parameters, include intrinsic clearance (Cl_{int}), enzyme affinity (K_m) and rate of reaction (V_{max}), plasma membrane permeabilities, renal and biliary excretion clearances, plasma protein binding, tissue-to-plasma partition coefficients (K_p), and absorption parameters. In most cases, data on these metabolic parameters can be obtained from *in vitro* studies (for example, by using hepatocytes and/or subcellular fractions).

The use of human cells and tissues

In drug development, it is particularly useful to be able to use human hepatocytes and/or microsomes, and the level of success achieved with such resources is encouraging. Ponsoda *et al.* (164) showed that the variability in human metabolism of several drugs in the clinic was reflected in the metabolism of these substances by hepatocytes isolated from the respective patients. However, inter-individual variation must always be taken into consideration, and there can be problems in obtaining suitable samples. Also, preparations can be susceptible to a wide range of factors, such as the solvents used (165), and the ability of different batches to attach to extracellular matrices (166).

ECVAM has recently funded a project (Contract No. 19471-2002-05F1ED ISP FR) to assess the possibility of obtaining high quality human hepatocytes in the USA and in the EU. The work resulted in the production of SOPs for obtaining preparations suitable for use as suspension or monolayer cultures. The problem of acquiring human liver cells is also being addressed (167, 168), and should be facilitated by the establishment of networks of human tissue banks for ethically-approved research, as opposed to other medical purposes (169).

***In vitro*–*in vivo* scaling**

The above factors, in conjunction with other pharmacokinetic parameters, such as plasma half-life, volume of distribution and oral bioavailability, together with the possibility of drug–drug interaction (170), have also been considered for the estimation of *in vivo* kinetics on the basis of *in vitro* studies in the process of *in vitro*–*in vivo* scaling. In addition, there are differences between the bioavailability of chemicals to target sites in *in vitro* systems, due to non-specific binding to plasma and tissue proteins (*in vivo*), as opposed to binding to components of tissue culture media (for example, serum protein) and microsomes *in vitro* (171, 172). The latter phenomenon has been suggested as being the reason why K_m values with hepatocytes are consistently lower than those obtained with microsomes, despite the fact that microsomes and hepatocytes inherently have similar capacities to carry out many CYP reactions (144).

Recent developments in applying biokinetic modelling

Significant advances are currently being made in biokinetic modelling, including the development of software programs and databases for the rapid generation of new models (George Loizou, personal communication; see <http://www.hsl.gov.uk/capabilities/pbpbk.htm>). These advances should improve the usefulness of this approach for evaluating large numbers of chemicals, and should assist in the interpretation of *in vitro* hazard predictions for risk assessment purposes.

Modelling target organ and systemic toxicity

Hepatotoxicity

Since it is the principal site for biotransformation within the body, the liver is the most often-used metabolising system, and, as would be expected, most hepatotoxicity studies have been conducted with freshly isolated hepatocytes. Many of the technical issues involved in using hepatocytes (for example, stability against dedifferentiation, including CYP inactivation during collagenase isolation techniques, and the induction of apoptosis [173, 174] and senescence, isolation and storage methods, criteria for viability, and methods for measuring enzyme activity and cell toxicity) are beginning to be overcome by using various techniques, such as the use of gel sandwich cultures and liver spheroids, and, more recently, there have been attempts to derive hepatocytes from adult stem cells (175–177; Vera Rogiers, personal communication).

Advances are being made in the use of histone deacetylase inhibitors to arrest the proliferation-related progressive loss of liver-specific functioning (dedifferentiation) and the spontaneous death of primary hepatocytes following their isolation from the liver, and to positively affect the maturation into hepatocytes of stem cells derived from human adult bone-marrow (178). Several potential sources of stem cells have been considered for generating differentiated hepatocytes, including embryonic stem cells and intra-hepatic progenitors. There have been some reports that, under appropriate culture conditions and with stimulation by cytokines, growth factors or chemical reagents, embryonic stem cells can differentiate into hepatocyte-like cells which express markers such as albumin and other plasma proteins, CK8/18, and glycogen, and permit the xenobiotic-mediated induction of CYP genes (179). Similarly, intra-hepatic progenitor cells have been shown to be able to generate hepatocyte-like cells expressing the same liver-specific markers. Ideally, the enzymes that are expressed by metabolically competent cells should reflect their relative *in vivo* importance for biotransformation. However, despite these advances, it is still not possible to routinely obtain differentiated hepatocytes from stem cells (179), although it is expected that this will be feasible in the near future.

Engl *et al.* (180) have shown that culturing human hepatocytes in three-dimensional collagen gel sandwich cultures improves the maintenance of specific functions, including metabolic competence. This is thought to be because the receptors for hepatocyte and epidermal growth factors are relatively easily activated. Human hepatocytes obtained from liver resections in this way have remained functionally active for up to 150 days in culture (181). However, Yuan *et al.* (182) have shown that human hepatocytes grown in monolayer two-dimensional cultures in the presence of 1% rat tail collagen, maintained the activities of a number of CYP isozymes and UDP-glucuronyltransferase over a two-week period, which raises questions about the need to use more-complicated culture systems. The ability to avoid the use of collagen gels for maintaining human hepatocytes would eliminate the problems of the variability in the attachment of freshly isolated hepatocytes from different donors that has been observed, in contrast to experience with rat hepatocytes, which attach readily (183).

There have been few efforts to validate the use of hepatocytes for the specific purpose of predicting toxicity. One study showed that primary rat hepatocytes could be used to predict the acute oral toxicity of a number of chemicals (184). However, this was a small investigation, conducted in a single laboratory. The workshop participants agreed that it is still not possible to recommend for formal prevalidation and validation, a standard protocol for using hepatocyte cultures for the specific purpose of pre-

dicting hepatotoxicity *in vivo* for regulatory purposes. The principal barrier to progress on this important issue appears to be the lack of suitable *in vivo* data on hepatotoxicity for benchmarking purposes, despite the recommendation in ECVAM Workshop 1 (held in 1994) that such a database should be established.

Modelling toxicity to extra-hepatic organs

It is difficult to model metabolism-mediated toxicity to other target organs *in vitro*, although it is possible to use subcellular metabolising fractions from different tissues with toxicity indicator cells and to demonstrate metabolic activation. The principal problem is that the specific activities of most extra-hepatic metabolising enzymes are usually several orders of magnitude lower than those of the corresponding enzymes found in the liver. Thus, this aspect of target organ toxicity is often overlooked, despite the fact that extra-hepatic metabolism might be extremely important when it is qualitatively different from that which occurs in the liver. Examples of situations where extra-hepatic biotransformation can be important are: a) oxidative metabolism by the intestinal mucosal lining (185), and reductive metabolism by the gut microflora (particularly relevant for investigating the toxicity of foods and food additives); and b) the prostaglandin H synthase (PHS) system (also known as the COX system). Metabolism by the intestinal mucosa, the gut microflora and the PHS system can all be taken into account in *in vitro* toxicity assays (29).

PHS catalyses the first two steps in the synthesis of prostaglandin, thromboxane and prostacyclin. Two forms of the enzyme have been characterised (PHS 1 and PHS2), both of which are haeme proteins that exhibit both cyclooxygenase and peroxidase activity, the latter being responsible for the one electron oxidation of a wide range of xenobiotics (186). The peroxidase activity of PHS can be expressed *in vitro*, if enzyme extracts are supplemented with arachidonic acid, and several chemicals are activated by the PHS system to become mutagens and carcinogens. In addition, extra-hepatic metabolism should be considered when it is necessary to model toxicity arising from exposure via other routes of entry, such as the skin (26) and the nasal epithelium (187).

Case Studies on Existing *In Vitro* Tests which Require Metabolism

Embryotoxicity

Reproductive toxicity testing has advanced through the development and successful validation of three

in vitro embryotoxicity tests. The results of the preliminary phase of the validation study were published in 2000, and the final results of the validation study have now been published (188). The tests involved the use of: post-implantation whole embryos; differentiating rat limb-bud cells in micro-mass cultures; and embryonic stem cells that retain totipotency, and the ability to be programmed to develop along a variety of specific differentiation pathways (189).

Embryotoxicity, particularly that which leads to teratogenicity, can be due to a variety of mechanisms, including the reactive binding of chemicals with nucleic acids and proteins, leading to mutagenesis and teratocarcinogenesis in the embryo, as well as the disruption of tubulin, microfilaments and the cytoskeleton, and many other subtle mechanisms that can affect development. It would be expected that the metabolites of certain chemicals would be responsible for some of the effects observed, and, *in vivo*, such metabolism occurs in the liver, the gonads, the placenta, and the embryo/fetus. The placenta has a relatively restricted complement of xenobiotic metabolising enzymes, but inducible CYP1A1 has been detected (190). The literature suggests that reproductive toxicity caused by metabolites is most likely to arise when exposure occurs during the adaptive responses caused by repeat dosing during the course of a reproductive toxicity study, a phenomenon due to the continued induction of the same metabolism enzymes, as would occur during any test involving chronic studies (15).

The inclusion of biotransformation systems in these tests would therefore be expected to improve their predictivity, as has been shown previously for chemicals, such as cyclophosphamide in the micro-mass assay (191), and also by using co-cultures of embryonic stem cells and metabolically competent cells (109). However, the ECVAM validation study was focused entirely on test chemicals not requiring metabolic activation, since the addition of metabolising systems might have complicated the standardisation of the *in vitro* embryotoxicity tests (192). Thus, the further use of these *in vitro* embryotoxicity tests will largely depend on how they can be adapted for the routine inclusion of a metabolising system for testing indirect-acting agents, and how relevant and reliable these modified protocols turn out to be. In fact, a group of experts recommended in 2003 that metabolic competence should be added to the EST (193).

For the testing of large numbers of chemicals, it should be noted that the EST is the only method which: a) could provide a relatively high throughput; and b) does not involve the killing of large numbers of pregnant animals. It is therefore recommended that an improved protocol for this test should be developed as a first priority for future validation, and there are plans in the recently initiated

EU ReProTect Project for further test development that involves incorporating metabolism, followed by a validation study.

Endocrine disruptors

A further example of where biotransformation is erroneously ignored in *in vitro* testing concerns endocrine disruptors (EDs; 114, 194). This is surprising, since: a) hormonally active chemicals, including some naturally occurring EDs, are known to be metabolised *in vivo*, some being activated; b) many potential EDs require metabolic activation to exert other toxicities; and c) the few studies available in which metabolising systems have been used, have shown that biotransformation can substantially modulate ED activity (195). One reason for the paucity of published studies involving the use of exogenous metabolism in *in vitro* assays for EDs is that potential EDs often bind non-specifically to proteins in the enzyme fractions (another situation where the application of *in vitro* biokinetic analysis would be particularly important, see earlier).

It is crucially important that *in vitro* methods for testing EDs should take account of the need to provide a biotransformation system. It would be preferable for this to involve cells that have been rendered metabolically competent, instead of adding exogenous enzyme fractions. This is because the use of cells would overcome the problem of binding to extracellular microsomal proteins. Moreover, if the cellular metabolising system expresses both phase I and II metabolism, it would also alleviate the problem of preferential activation *in vitro*, particularly as phase I ring hydroxylated products would be expected to be hormonally active, based on evidence from (Q)SAR studies (196), but would act as substrates for conjugation. It might even be feasible to generate genetically engineered mammalian cell lines containing steroid hormone nuclear receptors, their response elements and the associated reporter genes, together with genes expressing specific metabolising enzymes — all within the same cellular environment. Such multiple expressing cell lines could then be used to investigate the metabolic activation and detoxification of potential EDs *in vitro*. The possibility of developing such cell lines should be seriously investigated.

Acute toxicity testing

Acute toxicity testing is undertaken to investigate the effects of single large doses of a chemical. The workshop participants considered biotransformation to be likely to be less important as a mediator of such chemical toxicity, as compared with repeated dose toxicity. However, no consensus was reached on the relative importance of the many reasons why this could be so.

The results of the Multicentre Evaluation of *In Vitro* Cytotoxicity (MEIC) study showed good correlations between *in vitro* acute cytotoxicity data and human lethal blood concentrations for a diverse range of chemicals, and also with *in vivo* LD50 values from rodent studies (197). The data supported the hypothesis that basal cell cytotoxicity is a useful indicator of acute lethal systemic toxicity. It was further hypothesised that, since this endpoint is due to critical organ failure, particularly of the nervous system, such failure could arise from cell death or loss of function *in vivo*. Interestingly, the good correlation between basal cytotoxicity and acute toxicity was usually irrespective of cell type and assay, although the use of human cells understandably gave the best prediction of human lethal blood concentrations. However, there was no specific control of metabolism or incorporation of exogenous metabolising systems in the tests used, although these included human hepatoma cell lines and hepatocytes, which might have been expected to have possessed some metabolic competence. It is noteworthy that the level and nature of any such competence was not specifically reported.

Several of the test chemicals used in the MEIC study are known to be extensively metabolised to reactive forms, and in this respect it is notable that Halle (198) deliberately excluded, as far as possible, any IC50 values obtained with liver cell cultures (hepatocytes, hepatoma cell lines) from his registry of cytotoxicity, to avoid complications resulting from the possible metabolic activation or detoxification of chemicals.

Since the initiation of the MEIC study, it has been recognised that the predictivity of basal cytotoxicity assays for acute toxicity could be improved by various means, in particular, by including *in vitro* methods for biotransformation, absorption in the gut, passage across the blood–brain barrier, distribution volumes, protein binding, and renal clearance/accumulation in a test battery, as well as models for target organ toxicity, in conjunction with tests for neurotoxic potential (199). Clearly, such extra parameters in the testing battery should also take account of the use of human cells (200).

It is therefore to be welcomed that the recently initiated EU AcuteTox project is aimed at developing a testing strategy for using *in vitro* methods for acute toxicity prediction that will involve developing a system of alerts based on accounting for such known outliers of the correlation between acute systemic toxicity and basal cell cytotoxicity, primarily on the basis of biokinetics, metabolism and target organ specific effects.

Cell transformation

Cell transformation is the induction of certain phenotypic alterations in cells in tissue culture that are

characteristic of tumourigenic cells (201). Several different cell transformation assays have been devised, either involving rodent cell lines or immortalised fibroblast cell lines of rodent or human origin, and some systems comprise primary cells, such as those used in the SHE cell assay (202, 203). Some of these systems are used to detect initiators of carcinogenesis which act by a genotoxic mechanism via electrophilic interactions with cellular macromolecules such as DNA, while others can be used in a two-stage process to additionally detect tumour promoters via non-genotoxic mechanisms. Also, an *in vitro* cell transformation assay for tumour promoters has been developed, involving Bhas 42 cells derived from Balb/c 3T3 cells transfected with the *v-Ha-ras* oncogene (204).

In all the above cases, exogenous metabolising systems are not routinely used, on the assumption that any biotransformation necessary will be provided by the indicator cells themselves. However, this might not be the case, and the assays are inconsistently sensitive to carcinogens known to require activation. Moreover, when metabolising systems have been added, there have been some problems with the assays. This could be because: a) the test is lengthier than other *in vitro* genotoxicity assays; and b) some of the mechanisms involved in cell transformation operate over a longer time than those involved in genotoxicity. These factors are exacerbated by the technical problems encountered in attempting to maintain enzyme activity for a sufficiently long period of time.

However, it is known that SHE cells can express some metabolic competence (205), although Combes *et al.* (201) concluded that “*a thorough investigation is needed to ascertain the necessity of using exogenous metabolising systems in the various cell transformation assays.*” Unfortunately, such an investigation has not been conducted, and it is suggested that it should be part of the validation study of cell transformation assays being planned by ECVAM.

Conclusions

Prospects for using test systems incorporating metabolism

Apart from genotoxicity and hepatotoxicity studies, the incorporation of biotransformation into *in vitro* test systems has been inconsistent, despite the fact that biotransformation is known to play a key role in the toxicity of many chemicals. Since the residual metabolising capacity of indicator cells is often very low, inappropriate and variable, it is important to rectify this unsatisfactory situation in order to improve the predictivity of *in vitro* assays, particularly with respect to testing for sensitisation, cell

transformation, acute toxicity, target organ toxicity, endocrine disruption and embryotoxicity.

This will require further research to overcome some practical and logistical problems, and to identify the most appropriate ways of taking account of biotransformation. For example, a simple exogenous metabolising system, such as liver S9, cannot be used to screen chemicals for embryotoxicity *in vitro*, due to its own toxicity to the indicator cells. However, it might be possible to use metabolically-competent stem cells. Also, no consensus could be reached on whether, for mechanistic studies, the use of genetically engineered cell lines expressing key CYPs, and, in the case of embryotoxicity, other enzymes unique to the fetus, should be investigated. In fact, in embryotoxicity testing it is often assumed that the potential contribution of placental enzymes to metabolism is accounted for by the use of hepatocytes alone. In addition, strategies for screening chemicals for their susceptibility to biotransformation via specific mechanisms and pathways need to be developed and validated.

It is advisable that, as a matter of routine, the residual basal biotransformation competence of any *in vitro* test system should be characterised before it is developed and recommended for detecting toxicity. Moreover, the metabolic competence of indicator cells should be checked periodically to ensure that basal activities have not altered significantly. Lastly, the choice of metabolising system to use *in vitro* should be based on the purpose of the test. For example, selection might vary according to whether the test is a screen, or is to be used to provide hazard or mechanistic information, according to the generalised decision-tree scheme presented in this report (Figure 2).

Validation

It is clear that the validation of toxicity tests that incorporate metabolising systems will require the careful selection of chemicals for which there is unequivocal information on whether they are metabolically activated or detoxified (a list of potential chemicals for such a purpose is presented in Table 11). Such information should include the effects of human metabolism, since, ideally, where toxicity testing is being undertaken for human risk assessment, human-based biotransformation systems should be used. Metabolism-mediated toxicity data will be crucial, if such test systems are to be validated for regulatory use. Unfortunately, there is a lack of sufficient numbers of chemicals that meet this criterion, due to lack of information in the public domain. This is the reason why the workshop participants concluded that the validation of primary hepatocyte systems for detecting metabolism-mediated hepatotoxicity is not possible at the present time, although the systems can and should

be used for routine screening and research. Protocols for toxicity tests for metabolism-mediated toxicity will also need to include relevant positive and negative control chemicals that have known susceptibilities to activation or detoxification.

In silico methods for predicting metabolism and biokinetics

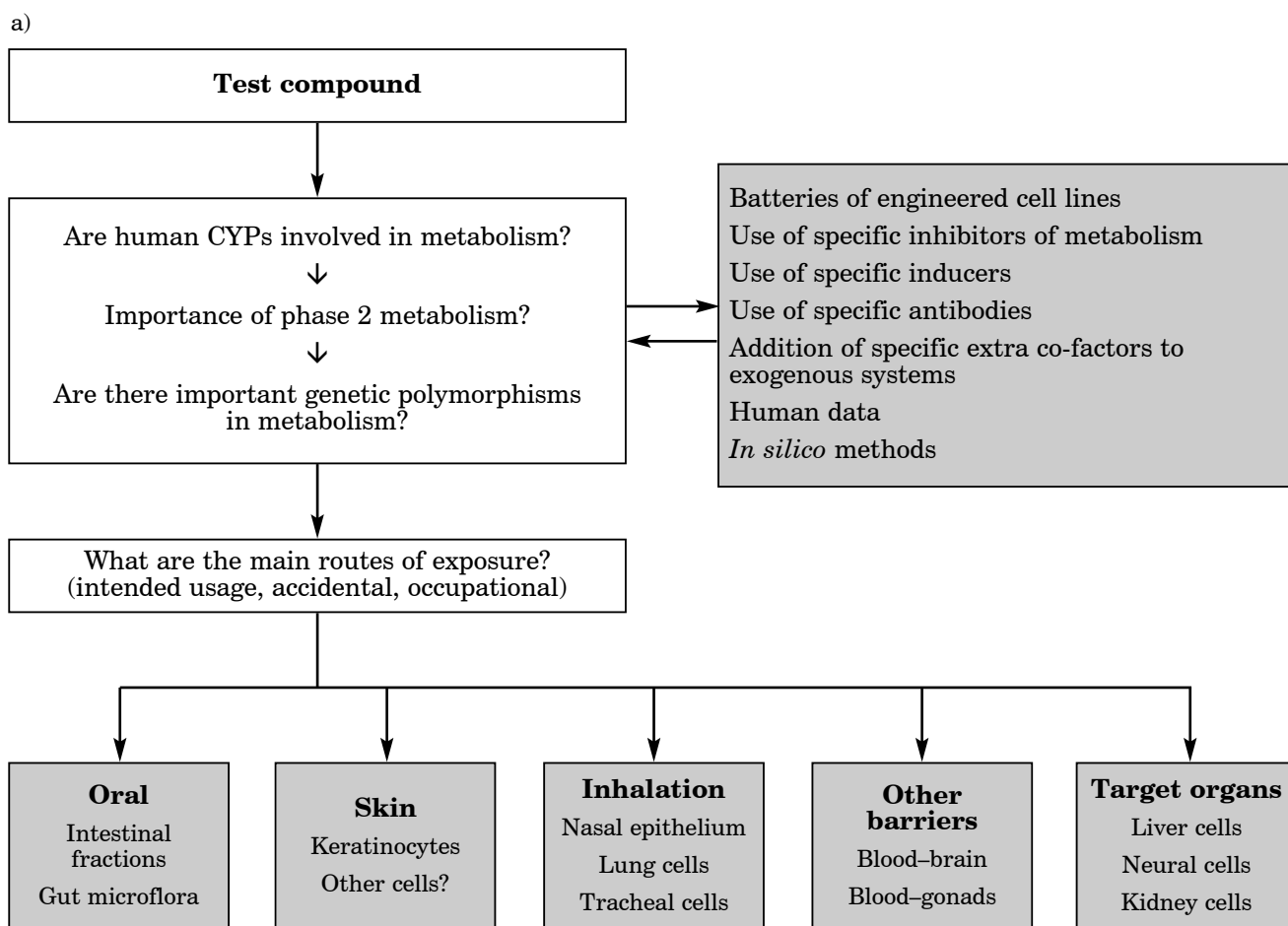
Status

Several *in silico* systems for predicting metabolism are available, including (Q)SAR models and expert systems, but none of these have been compared extensively for their relative performances, and none have been formally accepted for regulatory use, although some models can be used to provide supporting information in chemical risk assessments. There is currently no consensus on how *in*

silico models for predicting biotransformation should be validated. Also, a variety of systems are in different stages of development, assessment and validation. If they are to be of more practical use outside the pharmaceutical industry for regulatory testing, then further research needs to be undertaken to make them more amenable for a wider range of chemicals.

Problems with regard to the availability of good quality data for benchmarking purposes, apply to techniques for using *in silico* prediction systems and biokinetic models to assess the metabolic fate of chemicals after uptake by different routes of exposure in different species. Before these systems can be validated, more chemicals with good quality data need to be found, for use as test sets. Nevertheless, it was agreed that at least one biokinetic modelling system is, in principle, ready for more formal consideration for validation (206). Clearly, *in silico* systems for predicting toxicity should take account of the possibility that biotransformation could modu-

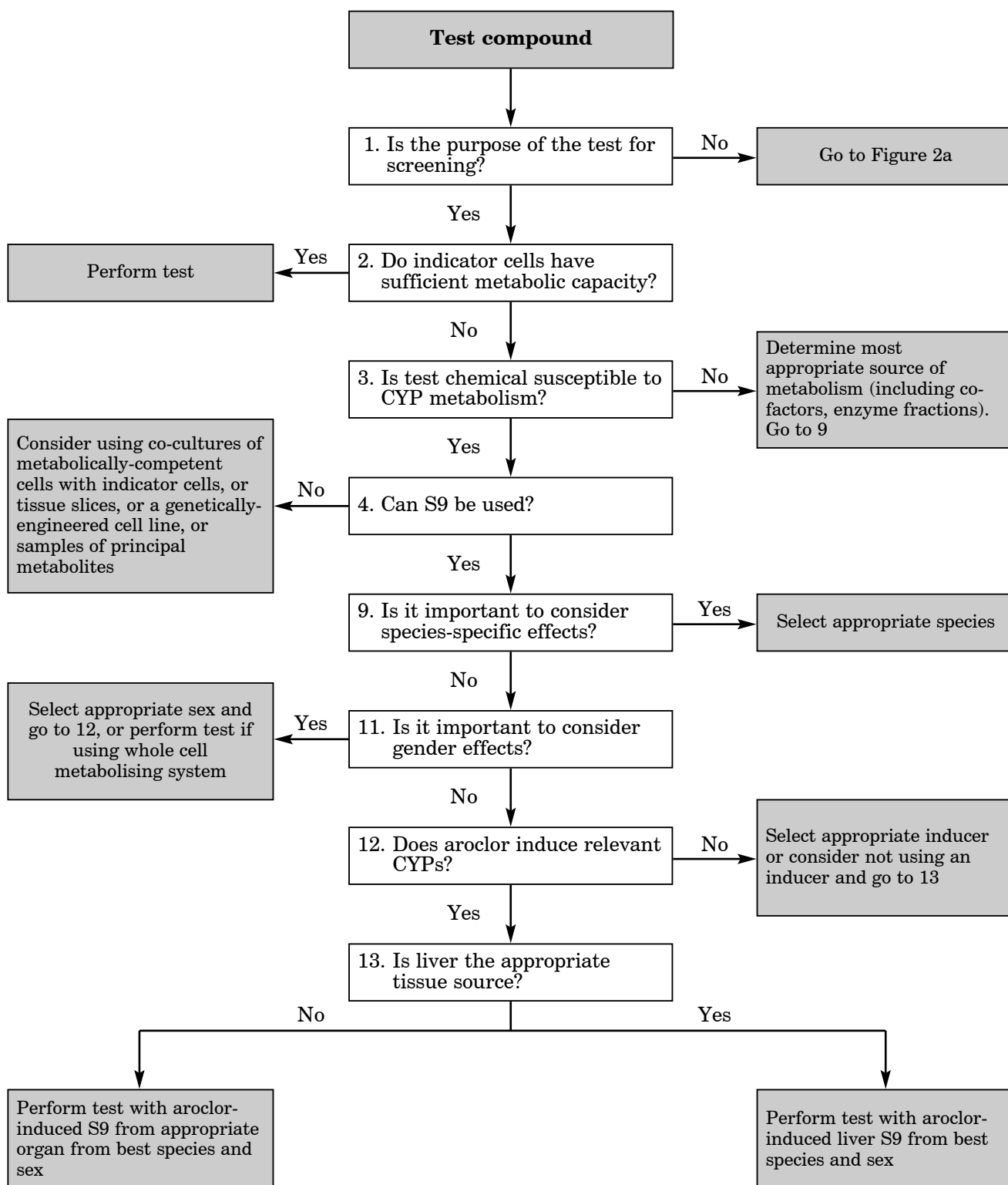
Figure 2: Schemes for incorporating metabolising systems



a) incorporating metabolising systems into in vitro assays for mechanistic studies.

Figure 2: continued

b)



b) incorporating metabolism into in vitro screening test systems.

Table 11: List of chemicals requiring metabolic activation, which are potentially useful for method development and validation studies^a

Acetaminophen
Albendazole
Amodiaquine
Bromobenzene
Carbamazepine

Clozapine
Cyclophosphamide
Dapsone
Ethinyl oestradiol
Flutamide

Furosemide
Ifosfamide
4-Ipomeanol
Iproniazid
Isoniazid

Lidocaine
3-Methylindole
N-[4-nitro-3-(trifluoromethyl)phenyl] acetamide
Phenacetin
Raloxifene

Troglitazone
Valproic acid
Vesnarinone
Ziprasidone

^aprepared by a workshop subgroup.

late toxicity. This could be achieved by modifying these systems, so that they can model the toxicity of the principal metabolites of chemicals, or by linking them with systems specifically designed to predict metabolite formation.

Relevance to *in vitro* testing

The bioavailability of chemicals to target sites, such as proteins, DNA or specific receptors in indicator cells, is an important determinant of toxicity in *in vitro* test systems. The bioavailability of test chemicals in *in vitro* assays (referred to as “*in vitro* biokinetics”) is affected by the nature of: a) the chemical being tested; b) the cells and the culture conditions; and c) the xenobiotic metabolising system (due both to the biotransformation of molecules and the non-specific binding to enzyme proteins). Such biokinetic analysis of *in vitro* test systems, by using similar approaches to those used to predict *in vivo* biokinetics, will enable target concentrations of chemicals applied to them to be

determined, and will therefore permit a more realistic assessment of the dose-response data obtained by using different *in vitro* and *in vivo* test methods for the same chemicals.

Recommendations

Method development and validation

1. A study should be commissioned to establish a database of chemicals that are toxic to humans, either with or without metabolic activation, and which are detoxified as a consequence of metabolism. It is suggested that information from existing databases, such as those maintained by IARC, ECETOC, RTEC, and IUCLID, should be assessed initially.
2. The above study should also include a comparison of animal and human toxicity models, with respect to the metabolite profiles and biokinetics of a range of chemicals.
3. An initiative should be undertaken to create a publicly available depository of reference chemicals that could be used in validation studies on biotransformation systems. The inclusion of a chemical in the depository should be based on a requirement for good quality animal and human toxicity data, which clearly demonstrate the role that biotransformation plays in the toxicity observed. Efforts should be made to balance the numbers of chemicals requiring, and not requiring, metabolic activation.

Biokinetic modelling

4. A workshop should be organised on *in vitro* biokinetics: a) to identify and discuss key methodological issues that need to be addressed, in order to facilitate the use of biotransformation systems in *in vitro* assays; b) to improve *in vitro* data interpretation and extrapolation; and c) to identify the analytical techniques required to elucidate the biokinetics of *in vitro* systems, with and without metabolism.
5. A project should be established with the objective of defining the proper use of physiologically-based biokinetic (= pharmacokinetic [PBPK]) modelling approaches for predicting ADME parameters.
6. The status of *in silico* systems for predicting metabolism, metabolites, metabolic pathways, and toxicity should be assessed by the ECVAM Task Force on Metabolism for their strengths

and limitations, and for their suitability for validation.

Incorporating biotransformation into toxicity tests

7. A study should be commissioned to develop a tiered xenobiotic metabolism strategy, designed to screen chemicals for their susceptibility to mammalian metabolism.
8. Methods for using and incorporating extrahepatic biotransformation systems into *in vitro* toxicity assays should be developed and further improved. Individual studies should be commissioned, to investigate: a) the metabolic competence of rodent and human cell lines used in cell transformation assays and ways to improve it; and b) the role of metabolism in non-genotoxic carcinogenesis.
9. Innovative, high throughput *in vitro* screening methods should be developed, in which several cell types can be tested in parallel studies by using multiplexed chemical, recombinant or biological metabolites. A particular effort should be made to characterise these *in vitro* cell-based assays, in order to understand the reaction of each individual cell (high content analyses) in each assay.

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Appendix

Data collection for the ECVAM SIS databases on experimental systems for studies in the fields of hepatotoxicity and metabolism mediated toxicity

Title	Method summary sheet ^a	Number of test results data sheets	Number of different test compounds
2001^b			
Preparation of precision-cut tissue slices	Yes		
Cold and cryopreservation of precision-cut liver slices	Yes		
Dynamic roller culture of precision-cut liver slices	Yes	376	97
Shaken submersion culture of precision-cut liver slices	Yes	231	80
Co-incubation of liver slices with other tissue slices	Yes	2	2
The use of liver slices for hepatotoxicity and metabolism-mediated toxicity studies	Yes		
2002^b			
Primary monolayer culture of isolated hepatocytes	Yes	1289	658
Primary monolayer culture of isolated hepatocytes	Yes	30 (for interlaboratory assessment study)	30
2003^b			
Three-dimensional hepatocyte culture as cell aggregates (spheroids)	Yes	13	12
Co-culture of primary hepatocytes with another cell type	Yes	15	13
Primary culture of isolated non-parenchymal liver cells	Yes	15	13
Collagen-gel sandwich configuration culture of primary hepatocytes	Yes	26	19
Monolayer culture of a liver cell line	Yes	634	419
2004^b			
Primary monolayer culture of isolated hepatocyte couplets	Yes	47	18
Suspension culture of isolated hepatocytes	Yes	557	431
2005^b			
Isolated perfused liver	Yes	645	101
Liver homogenate and subcellular fractions of liver tissue	Yes	134	116

^aMethod summary data sheet refers to the ECVAM SIS data sector, and does not necessarily mean that established methods are described; ^byear that information was added to the database.