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# Methods of in vitro toxicology

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**Summary**

In vitro methods are common and widely used for screening and ranking chemicals, and have also been taken into account sporadically for risk assessment purposes in the case of food additives. However, the range of food-associated compounds amenable to in vitro toxicology is considered much broader, comprising not only natural ingredients, including those from food preparation, but also compounds formed endogenously after exposure, permissible/authorised chemicals including additives, residues, supplements, chemicals from processing and packaging and contaminants. A major promise of in vitro systems is to obtain mechanism-derived information that is considered pivotal for adequate risk assessment. This paper critically reviews the entire process of risk assessment by in vitro toxicology, encompassing ongoing and future developments, with major emphasis on cytotoxicity, cellular responses, toxicokinetics, modelling, metabolism, cancer-related endpoints, developmental toxicity, prediction of allergenicity, and finally, development and application of biomarkers. It describes in depth the use of in vitro methods in strategies for characterising and predicting hazards to the human. Major weaknesses and strengths of these assay systems are addressed, together with some key issues concerning major research priorities to improve hazard identification and characterisation of food-associated chemicals.


*Keywords:* Hazard identification; Risk assessment; Food chemicals; In vitro methods; Biomarkers; Parallelogram approach

*Abbreviations:* ADI, acceptable daily intake; Ah, aryl hydrocarbon; BiP, immunoglobulin binding protein; CHEST, chicken embryo toxicity screening test; Comet test, single-cell microgel electrophoretic assay; DNA, deoxyribonucleic acid; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; ES, embryonic stem cells; EST, expressed sequence trap; EU, European Union; FETAX, frog embryo teratogenesis assay xenopus; FISH, fluorescence in situ hybridization; Grps, glucose-regulated proteins; GSH, glutathione; HESI, ILSI Human and Environmental Sciences Institute; Hsp, heat shock protein; HMWC, high molecular weight chemical; ICCVAM, US Interagency Committee for the Validation of Alternative Methods; IEF, isoelectric focusing; IgE, immunoglobulin; LMWC, low molecular weight chemical; LOEL, lowest-observed-effect level; MALDI, matrix assisted laser-desorption ionisation; MALDI-TOF, matrix assisted laser desorption-ionisation time of flight; MM, micromass; MTD, maximum tolerated dose; MT, metallothioneine; NRU, neutral red uptake; OECD, Organisation for Economic Cooperation and Development; PB-TK, physiologically-based toxicokinetic model; PCR, polymerase chain reaction; PCNA, proliferating cell nuclear antigen; PCs, partition coefficients; PMs, prediction models; QSAR, quantitative structure–activity relationship; RAST, radioallergosorbent test; mRNA, messenger-ribonucleic acid; ROS, reactive oxygen species; RSM, restriction site mutation assay; SAR, structure–activity relationship; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; SOP, standard operating procedure; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; $TC_{50}$, dose at which 50% of cells are affected; TTC, threshold of toxicological concern; Vd, apparent volume of distribution.

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**Contents**

## 1. Introduction

The use of non-animal test methods, including computer-based approaches and in vitro studies, provides important tools to enhance our understanding of hazardous effects by chemicals and for predicting these effects on humans (Broadhead and Combes, 2001). In vitro systems are used principally for screening purposes and for generating more comprehensive toxicological profiles. They are also of potential use for studying local or tissue and target specific effects. A major area of potential utility is to obtain mechanism-derived information. In vitro approaches are considered to be of additional value beyond 'Hazard identification' and hence it is important to consider their application to other elements of the risk assessment paradigm. Non-animal test methods, including computer-based approaches and in vitro assays, provide important tools to enhance the extrapolation from in vitro to in vivo in humans.

In vitro methods are widely utilised for screening and ranking of chemicals. In the case of food additives, in vitro data have already been considered in some instances for risk assessment purposes. However, in general, in vitro data have had no direct influence on the calculation of acceptable daily intake (ADI ) values, as reviewed by International Life Sciences Institute (ILSI) Europe (Walton et al., 1999). In vitro methods are invaluable in providing mechanistic information on toxicological findings both in experimental animals and in humans. It is anticipated that rapid advances in biomedical sciences will result in the development of a new generation of mechanism-based in vitro test strategies for hazard characterisation that can be applied in risk assessment. Therefore, it was felt that the subject of "Hazard identification by in vitro toxicology" cannot be adequately undertaken without also addressing other elements of the risk assessment paradigm. Hence this chapter addresses the state of the art, future potential, research needs and the hazard assessment of food-associated chemicals using established and novel in vitro toxicological approaches.

Food-associated compounds that can be investigated using methods of in vitro toxicology are: natural ingredients, including those from food preparation, compounds formed endogenously as a result of exposure, permissible/authorised chemicals including additives, residues, supplements, chemicals from processing and packaging and contaminants. Intrinsic limitations which are encountered in the in vitro assessment of toxicity by macronutrients and whole food, and in vitro approaches to study these aspects are discussed in section 2.3, on toxicokinetic modelling. One of the major future research needs is to develop new innovative technologies that will better enable the investigation of the absorption of individual food components from the gastrointestinal tract, their bioavailability as well as focusing on food matrix effects. Systems need to be developed to reliably model barrier functions (gastrointestinal tract, blood/brain) and to elucidate the role of transporter-proteins in cell membranes involved in absorption and efflux of compounds. Section 2.3 also provides an introductory review of the metabolic properties of in vitro systems, their characteristics and limitations, and which types of systems are available to provide metabolic activities relevant for the in vivo situation.

Cells respond rapidly to toxic stress by altering, for example, metabolic rates and cell growth or gene transcription controlling basic functions. The ultimate consequence termed "Cytotoxicity" is addressed in section 2.1. Cytotoxicity data are of their own intrinsic value in defining toxic effects (e.g. as an indicator of acute toxic effects in vivo) and are also important for designing more in-depth in vitro studies. One effect of reactive chemicals potentially encountered at subtoxic concentrations is the direct interaction with DNA that will result in various types of damage, including promutagenic lesions. Genetic lesions are not only a reflection of compound-induced events, but also indicators of genetic instabilities caused by DNA-repair deficiencies. The significance of these endpoints and of promutagenic lesions and other inherent non-genotoxic endpoints leading to cell transformation are presented in more detail in section 3.1 (Cancer-related endpoints).

The novel approaches of in vitro toxicology are, however, focused on the development of molecular markers based on detecting effects at levels of exposure

to potentially toxic chemicals lower than those that cause the onset of clinically observable pathological responses. Expression of stress response or other genes and ensuing biochemical alterations may be potential markers for compound-induced toxicity. In addition, the measurement of the transcription and translation products of gene expression can reveal valuable information about the potential toxicity profile of chemicals. The rapid progress in genomics and proteomics, in combination with the power of bioinformatics, creates a unique opportunity to form the basis of better hazard identification, for increasing understanding of underlying mechanisms and for a more relevant safety evaluation. The technologies that include DNA microarrays for transcriptome analysis and two-dimensional gel electrophoresis for proteomics are discussed, together with the functional responses in section 2.2 (Cellular responses). These methods are also of importance for identifying genetic polymorphisms, which represent an important factor of individual susceptibilities towards toxic compounds.

Methodologically, a major advance would be the introduction of relevant biomarkers for the identification of potential hazards of food chemicals and their metabolites formed in the body. Of equal importance is the critical utilisation of biomarkers for genetic susceptibility and for protection, factors of major importance in determining individual response. Levels of gene expression and genetic polymorphisms are important in biomarker studies and are discussed in section 4 (In vitro approaches for development of biomarkers). They are being used to find new endpoints by disclosing novel mechanisms of effect and to aid interpretation of biomarker results, for instance by providing basic information on how susceptibilities may influence the impact of risk factors.

Additionally, in vitro methods may provide a new generation of biomarkers, e.g. ex vivo challenge assays with lymphocytes, induction of functional responses in body fluids (blood plasma, urine, faecal water) of exposed humans, or determining toxicity parameters in isolated somatic cells from different tissues of potentially exposed humans. It is anticipated that it might become feasible in the near future to establish toxicity profiles as evidenced by transcriptomics/proteomics and by pattern analysis using appropriate comparative algorithms to predict at least acute/subacute toxicities and to identify known/unknown toxicity patterns.

An ambitious future aim is to predict chronic effects on the basis of in vitro studies. This would require the development of methods which can measure the course of molecular alterations that are also operative in long-term and complex sequences of events involved in chronic toxicity in vivo. Specific challenges will be encountered not only for prediction of cancer, but also for the prediction of other long-term target organ toxicities, such as lung fibrosis and toxic liver damage, nephrotoxicity, haematotoxicity and neurotoxicity. A problem to be solved is the organotypic cell hierarchy and tissue specific cell/cell or cell/matrix interactions. Solutions could include the development of longer-term tissue slice cultures, which are not yet sufficiently refined to be used as prediction assays. However, these potential solutions are not further addressed in this review due to the current limited information available in this area. Alternatively it may be possible to identify early pivotal events that can be markers of longer-term effects (see section 2.2, Cellular responses).

The different in vitro systems available to study developmental toxicity are discussed in section 4.2. Prediction of the effects on fertility resulting from low-level exposure to chemicals as encountered via the food chain is very challenging and still in its infancy. Moreover, as far as mammalian development is concerned, there is currently insufficient knowledge available of the full physiological and molecular developmental mechanisms. This limits the basis for an adequate understanding of toxic mechanisms and thus the development of predictive techniques.

Section 4.3 (Prediction of allergenicity) describes the types of complex immune responses that might arise as a consequence of exposure to chemicals, some of which may act as food allergens. Food-associated allergy is of considerable importance, since very small amounts of food components can elicit such responses, which can be acutely life-threatening in susceptible individuals. Moreover, food-related compounds can specifically target the gut-associated lymphoid tissue, the immune system associated with the gastrointestinal tract.

A prerequisite for the successful application of in vitro approaches is the availability of appropriate validated test systems (Balls et al., 1990, 1995; OECD, 1996; ICCVAM, 1997a). Validation independently establishes the reliability and relevance of a procedure or assay method for a specific purpose. Typically, it involves conducting an interlaboratory blind trial as a basis for assessing whether a test can be shown to be useful and reliable for a specific purpose according to predefined performance criteria. Validation studies are conducted principally to provide objective information on new tests, to confirm that they are robust and transferable between laboratories and to show that the data generated can be relied on for decision-making purposes. If a new test is to be endorsed as being scientifically valid, the outcome of the validation study must provide sufficient confidence in the precision and accuracy of the predictions made on the basis of the test results provided. The successful validation of a new toxicological test method is seen as a route to securing regulatory acceptance of that test (where appropriate), as well as being necessary to support the routine use of a new method.

The future paradigm should be to use appropriate, validated human-based test systems. The broad array of in vitro assays include: (a) subcellular systems, such as macromolecules, cell organelles, subcellular fractions; (b) cellular systems, such as primary cells, genetically modified cells, immortal cells, cells in different stages of transformation, cells in different stages of differentiation, stem cells, co-cultures of different cell types, barrier systems; and (c) whole tissues, including organotypic systems, perfused organs, slices and explants (e.g. limb buds or gut crypts). The systems need to be maintained according to the general rules of 'good cell culture practice' (Hartung and Gstraunthaler, 2000), and it is also necessary to adequately establish origin, quality and characteristics of the subcellular, cellular, tissue or organotypic systems used.

In addition to using appropriate in vitro toxicological systems to achieve an enhanced predictivity for hazards by food-associated chemicals, another major potential area of utility of in vitro methods lies in the development of mechanistic understanding of toxicological processes. By pursuing an intelligent development and application of new in vitro technologies, these systems can serve as a basis for a more targeted risk assessment of chemicals that in vivo toxicology cannot currently address adequately. In summary, it is generally accepted that in vitro assays are of intrinsic importance and are necessary per se to assess toxic activities of chemicals, and to help elaborate their mechanisms of action as well as aetiology of diseases.

## 2. In vitro assessment of general toxicity

### 2.1. Cytotoxicity

#### 2.1.1. Introduction

Cytotoxicity is considered primarily as the potential of a compound to induce cell death. Most in vitro cytotoxicity tests measure necrosis. However, an equally important mechanism of cell death is apoptosis, which requires different methods for its evaluation. The inhibition of apoptosis is also of toxicological importance (see section 4.1, Cancer-related endpoints). Furthermore, detailed studies on dose and time dependence of toxic effects to cells, together with the observation of effects on the cell cycle and their reversibility, can provide valuable information about mechanisms and type of toxicity, including necrosis, apoptosis or other events.

In vitro cytotoxicity tests are useful and necessary to define basal cytotoxicity, for example the intrinsic ability of a compound to cause cell death as a consequence of damage to basic cellular functions. Cytotoxicity tests are also necessary to define the concentration range for further and more detailed in vitro testing to provide meaningful information on parameters such as genotoxicity, induction of mutations or programmed cell death. By establishing the dose at which 50% of the cells are affected (i.e. $TC_{50}$), it is possible to compare quantitatively responses of single compounds in different systems or of several compounds in individual systems.

#### 2.1.2. State of the art

*2.1.2.1. The use of cytotoxicity data as a predictor of acute systemic toxicity*. Over the last two decades there has been considerable interest in using basal cytotoxicity data to predict the acute effects of compounds in vivo. If a compound is acutely toxic, it is anticipated that, in most cases, this reflects an insult to the intrinsic functions of cells. This approach has been successfully applied in a validated in vitro method to assess phototoxicity (Spielmann et al., 1998), based on ATP-dependent neutral red uptake into lysosomes (Borenfreund et al., 1988).

In a large study of a diverse range of chemicals, a reasonably good correlation was found between basal cytotoxicity and acute toxicity in animals and humans (Clemedson et al., 2000). Kinetic factors and target organ specificity of the toxic effect are major parameters compromising the correlation. For this reason basal cytotoxicity should be considered as a starting point in an integrated assessment of potential in vivo toxicity of food chemicals.

*2.1.2.2. Relevant endpoints*. The most frequently used endpoints in cellular toxicity testing are based on the breakdown of the cellular permeability barrier, reduced mitochondrial function (Borenfreund and Puerner, 1985; Werner et al., 1999), changes in cell morphology (Borenfreund and Borrero, 1984), and changes in cell replication (North-Root et al., 1982). These endpoints have been established for many years and in many cell types. Membrane permeability changes are measured by dye exclusion (trypan blue) or by the release of intracellular enzymes like lactate dehydrogenase (Decker and Lohmann-Matthes, 1988), preloaded $^{51}Cr$ (Holden et al., 1973), or nucleoside release (Thelestam and Molby, 1976), uridine uptake (Shopsis and Sathe, 1984; Valentin et al., 2000), or vital dye uptake (Garret et al., 1981). Other tests rely on determination of cellular protein content (Balls and Bridges, 1984; Dierickx, 1989) or plating efficiency (Acosta et al., 1980; Strom et al., 1983). However, for differentiation between cytotoxicity and reversible cell damage, recovery of the cell needs to be appropriately considered and this might help interpret results of studies in vivo (Valentin et al., 2000) (see also section 2.2, Cellular responses). Apoptosis can be evaluated using changes in cell morphology, membrane rearrangements, DNA fragmentation, caspase activation, cytochrome c release from mitochondria, etc. It should be noted that this is a rapidly evolving field with obvious relevance to toxicology (see also section 2.2, Cellular responses).

*2.1.3. New developments and research gaps*

Differential biotransformation capacity can be very important. Most cellular systems have only poor metabolic competence and therefore cannot necessarily be considered as satisfactory models for the in vivo situation (see section 2.3, Toxicokinetic modelling and metabolism). Cytotoxicity testing can be refined by considering the target organ of the test compound in vivo and selecting a cell system that is appropriate on the basis of metabolic competence and of organ/tissue specific cytotoxicity (see section 3, Endpoints of in vitro toxicology testing). Increasingly, genetic engineering is improving the utility of cells in such studies.

*2.2. Cellular responses*

*2.2.1. Genomics, transcriptomics and proteomics*

*2.2.1.1. Introduction*. The basic methodology of safety evaluation has changed little during the past decades. Toxicity in laboratory animals has been evaluated by principally using clinical chemistry, haematological and histological parameters as indicators of organ damage. The effect of a toxic chemical on a biological system in most cases is fundamentally reflected, at the cellular level, by its impact on gene expression. Consequently, measurement of the transcription (*mRNA*) and translation (protein) products of gene expression can reveal valuable information about the potential toxicity of chemicals before the development of a toxic/pathological response.

The rapid progress in genomic (DNA sequence), transcriptomic (gene expression) and proteomic (the study of proteins expressed by a genome, tissue or cell) technologies, in combination with the ever-increasing power of bioinformatics, creates a unique opportunity to form the basis of improved hazard identification for more predictive safety evaluation.

*2.2.1.2. State of the art — the technologies of genomics, transcriptomics, proteomics and bioinformatics*

*2.2.1.2.1. Genomics and transcriptomics*. The term 'genomics' is used to encompass many different technologies, all of which are related in some way to the information content of a cell (i.e. its DNA or RNA), which is based on the central dogma of molecular biology in which genes encoded in DNA are copied into messenger RNA (mRNA), which is then translated into functional proteins. Currently, there are two main approaches to the analysis of molecular expression patterns: (1) the generation of mRNA expression maps (transcriptomics); and (2) examination of the 'proteome', in which the expression profile of proteins is analysed. Classical approaches to transcript profiling such as Northern blotting, RNase protection assays, S1 nuclease analysis, plaque hybridisation and slot blots are time consuming and material intensive ways to analyse mRNA expression patterns. mRNA transcripts can also be analysed using 'real time' polymerase chain reaction (PCR) after reverse transcription. The advantage of this technique is that it provides a quantitative measure of individual mRNAs (Heid et al., 1996). Several companies provide systems for real time PCR; however, a limitation is the need to design primers specific for the genes of interest. Using these methods, an investigator is typically restricted to studying a limited number of genes at a time. For these reasons, enormous effort has been undertaken to develop methods that are both quantitative and allow analysis of thousands of genes simultaneously. Newer methods such as differential display (Liang and Pardee, 1992), serial analysis of gene expression (SAGE) (Velculescu et al., 1995), and most significantly, the development of cDNA/oligonucleotide microarrays (Schena et al., 1995; Zhao et al., 1995; DeRisi et al., 1996) offer unprecedented power for use in a wide range of scientific disciplines. Table 1 outlines currently available methods for the study of gene expression at the transcript level.

*2.2.1.2.2. DNA/oligonucleotide microarrays*. A completely different approach to the study of gene expression profiles and genome composition has been developed with the introduction of DNA/oligonucleotide microarrays (Watson et al., 1998; Duggan et al., 1999; Graves, 1999), which allows the simultaneous semiquantitative measurement of the transcriptional activity of thousands of genes in a biological sample. Such microarrays are generated by immobilising cDNAs, PCR products or cloned DNA, onto a solid support such as nylon filters, glass slides or silicon chips (Bowtell, 1999). DNA arrays can also be assembled from synthetic oligonucleotides, either by directly applying the synthesized oligonucleotides or by a method that combines photolithography and solid-phase chemical synthesis (Fodor et al., 1993). The genes represented on the array can be chosen to cover specific endpoints or pathways or may include genes, which cover a wide range of biological processes. To determine differences in gene expression, a fluorescently or radioactively ($^{32}P$ or $^{33}P$)-labelled RNA probe(s) (normally a cDNA copy) is hybridised to the DNA or oligomer-carrying arrays. The surface is either scanned with a laser (and the fluorescent signal at each feature element is recorded) or for radioactively labelled probes, the signal is visualised using phosphorimager analysis or autoradiography. By these approaches, the expression of 10,000 genes or more can currently be analysed on a single array (i.e. a single experiment) and the relative changes in gene expression between two or more biological samples can be measured (Brown and Botstein, 1999). Development of this technology has been possible only through the efforts of the human genome project (and sequencing

projects for other organisms), where thousands of gene sequences have been determined and published. Numerous commercially available nucleic acid arrays using a range of different platforms are now widely available or in development.

In an analogous manner to the development of recombinant DNA and PCR, microarrays already have a large number of applications that will expand and diversify over time including: large-scale gene expression profiling; gene mapping and identification; tissue profiling of gene expression; mechanistic insight into broad range of biological processes; new markers of disease susceptibility; identification of potential new drug targets; platform for drug screening and toxicology studies; detection of mutations and polymorphisms; application to knockout technology; positional cloning of disease genes; evolutionary sequence analysis and pathogen identification.

*2.2.1.2.3. Proteomics.* For many years it has been possible to array complex protein mixtures by two-dimensional gel electrophoresis, which combines separation of proteins by isoelectric focusing (IEF) in the first dimension followed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) based on molecular weight in the second dimension. The product is a rectangular pattern of protein spots that are typically revealed by Coomassie blue, silver or fluorescent staining. However, the amount of each protein separated is at least one order of magnitude below the amount needed for chemical characterization (O'Farrell, 1975) and therefore the development of semi-preparative methods for purifying proteins in parallel and more sensitive techniques for protein characterization were needed (Vorm and Mann, 1994). Currently, techniques based on mass spectrometry (MS) are driving the progress in proteomics, the study of proteins expressed by a genome (simple organism), tissue or cell (Williams and Hochstrasser, 1997), in particular the introduction of MALDI (Matrix-Assisted Laser-Desorption/Ionisation) greatly expands the range of proteins that can be analysed with MS. Protein mass fingerprinting which is a fast and efficient way to identify proteins from MS, was subsequently introduced in 1993. This method involves selectively cutting proteins with an enzyme, usually trypsin, and comparing the fragment masses to theoretical peptides, similarly 'digested' by the computer, from databases. For peptide mass-fingerprinting MALDI-TOF (Matrix-Assisted Laser-Desorption/Ionisation Time Of Flight) is usually used. Both this method and electrospray can detect low levels of protein and are suitable for automation. In the case of MALDI-TOF, several thousand proteins isolated from gels may be analysed per week. It is now possible to conceive of a complete description (following further technological developments) at the protein level of an organism, tissue or cell under a given set of conditions. Unlike the genome of an organism, which is essentially fixed information underpinning the organism, the proteome (and likewise the transcriptome) is a varying feature subject to changes due to developmental stage, disease state or environmental conditions and is therefore closer to the biological consequences of altered gene expression. Proteomics thus benefits from the wealth of information accumulated by genome-based approaches, which allow peptide data to be connected directly to nucleotide sequences and gene information.

*2.2.1.2.4. Bioinformatics.* Bioinformatics, which in the simplest sense can be described as the interface between biological and computation sciences, is a key requirement for the organisation, analysis and storage of the voluminous quantities of data generated by the use of genomic, transcriptomic and proteomic technologies. Distilling information from complex DNA microarray or proteomic data demands sophisticated bioinformatic tools.

The key to full interpretation of results from genomic, transcriptomic and proteomic analysis is the integration

Table 1
Current methods for the study of gene expression at the transcript level

| | |
|---|---|
| Hybridization-based techniques | Northern blotting |
| | S1-mapping/RNase protection |
| | Differential plaque hybridization |
| | Subtraction cloning |
| | DNA microarrays |
| PCR-based techniques | Differential display |
| | RDA (representational difference analysis) |
| | Quantitative (real time) PCR |
| Sequence-based techniques | ESTs (expressed sequence tags) |
| | SAGE (serial analysis of gene expression) |
| | MPSS (massively parallel signature sequencing) |
| | DNA-sequencing chip |
| | Mass-spectrometry sequencing |

of information from different sources, linking gene expression data to DNA sequence information, experimental data available in public databases with the ultimate aim of detecting pathways and sets of genes tightly correlated with specific endpoints of toxicity.

Data mining techniques that aim to identify (analytically) trends and patterns in large data sets that would remain hidden from standard statistical analysis techniques are now being applied to DNA microarray data. Data mining applications are built on complex algorithms that derive explanatory and predictive models from larger sets of complex data by identifying patterns in data and developing probable relationships. For example, pattern recognition can be used to identify groups of genes that are regulated in a similar way across many experiments, or groups of treatments that provoke similar transcriptional responses in many genes. Similarities can be calculated using suitable statistical methods and displayed in a format that allows visual identification of gene expression patterns. For example, powerful clustering algorithms can be used to provide information on genes with a similar expression pattern (Eisen et al., 1998).

*2.2.1.3. Applications of transcriptomics and proteomics to hazard identification*

*2.2.1.3.1. Transcriptomics applied to toxicological hazard identification.* The application of genomics/transcriptomics to toxicology (toxicogenomics) has the potential to have a huge impact on our ability to characterise compounds with the potential for adverse health effects by offering a more effective way to identify toxic hazards, which will form the basis of more predictive safety evaluation. In addition, it will greatly improve our current understanding of the mechanisms of toxic processes. Among other applications, it is likely to be useful in investigating the toxicological effects of food chemicals. The principles surrounding the application of global gene expression analysis to toxicology are based on the fact that almost without exception, gene expression changes will occur during toxicity, either as a direct or indirect result of toxicant exposure. These changes in gene expression are often a more sensitive, characteristic and measurable (at subtoxic doses) endpoint than the toxicity itself and provide novel information to complement and refine established methods. The use of these technologies to analyse global changes in gene expression may permit the identification of diagnostic gene expression patterns, which can then be used to determine the toxic potential of agents (at subtoxic doses and early exposure time points). In addition, they may provide new markers of toxicity (see section 4, Biomarker development) and will allow enhanced extrapolation between experimental animals, humans and human in vitro models in the context of hazard identification, to allow the development of more relevant, mechanistically-based in vitro systems.

One of the challenges facing toxicologists is to establish, under a defined set of experimental conditions, the characteristic pattern of gene expression elicited by a given toxicant and to compare this to data collected for known toxins acting via the same mechanisms. As the database of 'known toxins' grows for an individual toxic mechanism, it may be possible to develop 'mini-arrays' customised for specific toxic endpoint detection, based on pattern recognition. Microarray technology offers an ideal platform for this type of analysis and could provide a novel approach to toxicology testing.

A number of laboratories [e.g. National Institute of Environmental Health Sciences (NIEHS) (Nuwaysir et al., 1999)] and commercial companies have now assembled 'toxicology specific microarrays' from a range of species including rat, mouse and human containing genes which have previously been shown to be implicated in responses to toxicological insults. Thus, these arrays, which identify alterations in expression of toxicologically important genes (molecular fingerprints) could not only point to the possible toxicity of chemicals but also aid in elucidating their mechanisms of action through identification of gene expression networks. They allow greater sensitivity in detecting the effects of harmful compounds, and at the same time reduce the amount of time needed to understand how these compounds affect biological systems. The use of microarrays in toxicological risk assessment offers many significant advantages. For example, it may reduce the dependence on animals for toxicological studies in a number of ways. Screening with a microarray when used to complement a bioassay may enable doses to be lowered to a level that more closely resembles typical human exposure levels. Microarrays may help in exploring the connection between acute and chronic toxicity and identify secondary effects by studying the relationship between the length of exposure and the gene expression profiles generated by that toxicant. This could mean shorter bioassays, more realistic test dosages and considerable savings when compared with more traditional assays.

*2.2.1.3.2. Proteomics applied to toxicological hazard identification.* Toxicology is likely to prove one of the most important applications of proteomics. 2D-gel electrophoresis is a highly sensitive means of screening for toxicity and probing toxic mechanisms. By comparing proteins expressed following exposure of a biological test system to a chemical with those present under untreated conditions, it is possible to identify changes in biochemical pathways via observed alterations in sets of proteins that may be related to the toxicity. Once a large library of proteomic signatures has been compiled for compounds of known toxicity, it will be possible to use it to assess the toxicity of compounds whose toxicity is not known. One of the significant advantages of

proteomics is the ability to analyse proteins using high throughput, automated techniques that can be applied to the analysis of tissue samples, cell cultures and also body fluids (e.g. serum, urine, cerebro-spinal fluid, synovial fluid) suggesting that proteomics has great potential as a screen for new markers of toxicity and exposure. For example, it could have great potential as a method for detecting early markers of changes in humans resulting from continuous exposure to specific agents (see section 4, Biomarker development). To date, few large-scale proteomics studies have yet entered the public domain, therefore the full potential of the use of proteomics in toxicology has yet to be realised.

*2.2.1.3.3. Transcriptomics and proteomics — a combined approach*. The combination of transcriptomics and proteomics provides a very powerful tool for detecting early changes in toxicity and should be considered as complementary technologies. For example, low abundant transcripts may not be easily quantified at the protein level using standard 2D-gel electrophoresis. The expression of such genes may be preferably quantified at the mRNA level using techniques allowing PCR-mediated target amplification. Tissue biopsy samples as well as cell culture models typically yield good quality of both mRNA and proteins. However, the quality of mRNA isolated from body fluids is often poor due to the faster degradation of mRNA when compared with proteins. RNA samples from body fluids such as serum or urine are often not very meaningful markers for toxicity, and secreted proteins are more likely to be more suitable surrogate markers for toxicity. Detection of post-translational modifications, events often related to function or non-function of a protein, is restricted to protein expression analysis. The growing evidence of a poor correlation between mRNA and protein abundance (Anderson and Seilhamer, 1997) further suggests that the two approaches, mRNA and protein profiling, are complimentary and should be applied in parallel.

*2.2.1.4. Challenges and research gaps*

*2.2.1.4.1. Microarray technology and experimental procedures*. Microarray technology is still in its infancy and further improvements to the technology with respect to reproducibility, speed, cost and sensitivity will be needed. In addition, there is no current consensus for standard procedure concerning quantitation and interpretation of the large volumes of data produced. A significant number of questions remains regarding both the experimental protocol (e.g. how many time points? how many/what doses? which in vitro model?) and the gene expression data (e.g. how many genes should be measured and which ones? what is the relationship between gene expression patterns and toxic endpoints? is expression of certain genes always indicative of undesirable effects? are there gene expression thresholds beyond which a compound is toxic? how do we interpret gene changes for genes with different dynamic ranges?). These points illustrate the enormous amount of work required to understand the power and limitations of microarray technology, and some of the large collaborative activities currently taking place, such as the ILSI Human and Environmental Sciences Institute (HESI) Subcommittee on the Application of Genomics and Proteomics to Mechanism-Based Risk Assessment, will start to address these issues.

In addition, if humans are to be analysed, then the extent to which the global gene expression pattern varies between individuals, both before and after toxicant exposure, as well as the effects of age, diet and other factors on this expression needs to be determined. Experience in the form of large datasets will start to address and answer these questions.

*2.2.1.4.2. Proteomic technology*. Despite its potential, there are still technical limitations with proteomic technology. For example, only a limited proportion of the proteins can be extracted using current procedures. Visualisation of the resolved proteins also needs further development, since some low abundance proteins such as transcription factors may not be detected. Within these limitations, proteomics offers results that relate more directly to functionality, making proteomics particularly attractive for the analysis of complex protein mixtures from any cell type.

*2.2.1.4.3. Data handling and interpretation — bioinformatics*. One of the most significant challenges for the application of DNA microarrays is the interpretation of data. Cross-referencing results from multiple experiments (time, dose, replicates, different animals/species) to identify commonly expressed genes is itself a great challenge. Thousands of data points can be generated from a single experiment which therefore requires specialised software to analyse the computer output of image files, combined with sophisticated software that has clustering algorithms (Eisen et al., 1998) to allow the determination of patterns of gene expression changes across doses, time points, etc. This approach will have useful applications in sorting array data on different compounds with a similar toxic endpoint, facilitating the identification of diagnostic patterns of gene expression for these endpoints. In turn, these approaches should enhance prediction of the toxicity of novel compounds.

*2.2.1.4.4. Building reference datasets and correlation with classic endpoints of toxicity*. In order to fully interpret the results from transcriptomics and proteomics experiments for chemical hazard identification, it will be necessary to develop databases of known effects from chemicals with well-characterised toxic effects. In

addition, this is further complicated by attempting to correlate gene expression data to more classic endpoints of toxicity—a very challenging but necessary step in helping to evaluate and understand adequately the biological significance of the observed gene expression changes. Gene expression changes do not imply that the chemical will exert a toxic effect per se on long-term exposure at relevant dose levels. Initiatives such as the ILSI Human and Environmental Sciences Institute (HESI) Subcommittee on the Application of Genomics and Proteomics to Mechanism-Based Risk Assessment will start to address these issues for specific toxic endpoints with selected chemicals.

*2.2.1.5. Conclusions and future priorities*. The basic methodology of safety evaluation has changed little during the past decades. Toxicity in laboratory animals has been evaluated by using clinical chemistry, haematological and histological parameters as indicators of organ damage. The rapid progress in genomics, transcriptomics and proteomics, in combination with the ever-increasing power of bioinformatics, creates a unique opportunity to improve the predictive power of safety assessment by offering a more effective way to identify toxic hazards.

There are some issues that need to be addressed before the full potential of these technologies applied to toxicological hazard identification can be realized. Among these are the selection of model systems, dose selection, duration of exposure and the temporal nature of gene expression. We also need to have an understanding of how variable global gene expression patterns can be between different individuals, both before and after toxicant exposure. Numerous factors such as age, diet and other environmental factors may have significant effects on the gene expression profiles of different populations/individuals.

One way of starting to address these issues will be in the development of publicly available databases of gene and protein expression profiles from standardized test systems following exposure to well-characterised toxicants under defined experimental conditions and to relate these changes to other measures of toxicity, for example histopathology and clinical chemistry parameters.

The proliferation of different microarray platforms, while continuing to aid the evolution of the technology, may create problems of data comparison between platforms. In addition, the large variety of experimental conditions under which different laboratories will collect data will make large-scale data analysis a significant challenge. As a starting point in addressing this issue, the ILSI HESI Subcommittee on the Application of Genomics and Proteomics to Mechanism-Based Risk Assessment has as one of its objectives, a large-scale cross-platform, cross-laboratory experimental programme. Hopefully, it will be possible, with properly designed and controlled experiments, to compare results from these types of studies between laboratories.

If transcriptomics and proteomics are to realise their full potential in the development of novel approaches to toxicological testing, and in addition, contribute to the replacement of animal tests, then sufficient attention needs to be paid to the development of relevant biological model systems. For example, while cultured cell systems have many practical advantages, availability of suitable and sensitive cell lines/models with relevance to humans, and having metabolic competence, required to produce the toxic metabolite are important considerations. Transcriptomics and proteomics (through the application of pattern recognition) could also play a valuable role in the characterisation (including the identification of polymorphisms) of cells for the development of in vitro human model systems. This will be particularly important for the development and characterisation of suitable long-term in vitro systems for predicting the chronic effects of chemicals contained in foods.

Although many issues remain to be resolved and significant challenges lie ahead, it is clear that information obtained from genomics, transcriptomics and proteomics will have a significant impact on the approach to toxicology in the future. One can predict that the information gathered from experiments using these technologies will form the basis for improved methods to assess the impact of chemicals on human health.

*2.2.2. Functional responses*

*2.2.2.1. Introduction*. The development of molecular markers of subtle effects of potentially toxic chemicals that occur before the development of frank toxicity/pathology could have widespread use as indicators of toxicant response both in vitro and in vivo (see section 5, Biomarker development). Cells from various organisms respond rapidly to toxic stress by altering, for example, metabolic rates, and cell growth or gene transcription controlling basic functions. Stress or a change in gene expression may be potential markers of chemically-induced toxicity. There is considerable evidence indicating that many stress responses occur before any measurable cytotoxicity, thus allowing the monitoring of stress pathways at subtoxic levels. Such markers could provide an early, sensitive and reproducible response for monitoring the potential toxicity of chemicals in food.

*2.2.2.2. State of the art — cellular responses as early markers of toxicity*. Over the last few decades, a large amount of research has resulted in an explosion of information regarding mechanisms of toxicity and new tools to study the biological responses to toxic stress. None the less, major questions remain regarding mechanisms of cell injury and our ability to predict toxicity remains a significant challenge. The application

of molecular technology has opened new areas of exploration in toxicology and in particular, advances in gene and protein expression technologies provide the means to profile expression of thousands of messenger RNAs or proteins (see section 2.2.1, Genomics, transcriptomics and proteomics). Thus, there is great potential to provide new and better measures of cellular injury. Despite the tremendous volume of data being collected on gene expression during toxic stress, the number of genes for which activation has been linked to mechanism is small. In an ideal application, activation of a gene would signal a specific biological or biochemical response relevant to a mechanism of toxicity. Cells from various organisms respond to the presence of toxic chemicals via a number of different biochemical mechanisms. In most cases, this is related to the structure of the chemical, and therefore initial consideration of the structure of the compounds needs to be made in order to select the most relevant endpoint for investigation. Some of the key cellular responses to toxicant exposure, which could potentially be used as early markers of toxicity include the following:

a) Responses following exposure to toxicants that form reactive electrophiles (e.g. oxidative stress) such as loss of glutathione (GSH), increased production and sensitivity to reactive oxygen species (ROS), increase in cellular calcium, lipid peroxidation, loss of ATP and mitochondrial/endoplasmic reticulum (ER) specific events.
b) The cellular response to stress, including an increase in synthesis of the heat shock (Hsp) family of proteins, induction of the stress-activated protein kinases (SAPKs) and glucose-regulated proteins (Grps).
c) Changes in the levels of key enzymes, such as the phase I and phase II metabolising enzymes involved in the detoxification of toxic chemicals.
d) Induction of the metal-binding proteins, metallothioneins (MTs).
e) Perturbations to cellular membranes, gap junctions and intercellular communication inhibition (involving the connexins Cx43, Cx32 and Cx26).
f) Induction of cell proliferation (for which suitable markers could include TNF-α, TNF-β, plasminogen activator inhibitor-2 (PAI-2), the tumour proliferative marker Ki-67 antigen and proliferating cell nuclear antigen (PCNA).

*2.2.2.2.1. Genotoxicity (see section 3.1.2, Genotoxicity).* This list is by no means exhaustive, but illustrates some of the key biochemical events following exposure of a cell to a toxic chemical (the exact nature of the response depending on the nature of the chemical and the cell type). A number of these responses may be useful intermediate markers prior to the detection of acute toxicity. In addition, it is possible that cellular response patterns, generated by the use of transcriptomics and proteomics technologies could be predictive of chronic effects, following establishment of databases of known toxicities (initially in vivo and subsequently correlating effects found in vitro). This would provide a rational mechanistic approach to the in vitro identification of chronic toxicity. Finally, cellular responses, identified through the use of transcriptomics and proteomics could be helpful in understanding age-related effects by providing a basis (global expression patterns) from which to interpret age-related changes.

A few of these cellular responses are described below in more detail, with examples to illustrate their potential as early markers of chemically induced toxicity.

*2.2.2.2.2. Oxidative stress and glutathione homeostasis.* Increases in the intracellular levels of ROS, frequently referred to as oxidative stress, represents a potentially toxic insult, which if not counteracted will lead to membrane dysfunction, lipid peroxidation, DNA damage and inactivation of proteins. These endpoints are often used as endpoints in the study of oxidative stress. The ideal situation would be where a particular biological or biochemical response relevant to a mechanism of toxicity was reflected by changes in the expression of a specific gene or genes. Xenobiotics comprise one source of ROS because some xenobiotics can enhance the production of oxyradicals within the cells. Quinones, some dyes, bipyridiyl herbicides, some transition metals and aromatic nitro compounds comprise classes of compounds known to redox cycle. The detection of lipid peroxidation as an endpoint is currently complicated and the assays available can give only a crude estimation of the extent of lipid peroxidation. To prevent damage to cellular components, there are numerous enzymatic antioxidant defences designed to scavenge ROS in the cell. Examples include the superoxide dismutases (SODs), catalase, glutathione peroxidase (GPx), glutathione reductase (GR). Measurement of levels of these enzymes could provide a marker of oxidative stress. In addition, some of the genes/proteins induced in response to oxidative stress which could have potential as markers of oxidative damage include: NF-κB, cyclo-oxygenase-2 (COX-2), the transcription factor early growth response (Egr-1), c-fos, c-jun, c-myc, c-jun $NH_2$-terminal kinase (JNK), inducible nitric oxide synthase (iNOS), interleukin 8 (IL-8), intercellular adhesion molecule-1 (ICAM-1) and thioredoxin (Trx).

Glutathione-associated metabolism is a major mechanism for cellular protection against agents, which generate oxidative stress. GSH participates in detoxification at several levels and may scavenge free radicals, reduce peroxides or be conjugated with electrophilic compounds. Thus, GSH provides the cell with multiple

defences not only against ROS but also against their toxic products. The depletion of GSH during oxidative stress has a significant impact on the antioxidant capacity within a cell and provides a suitable marker for oxidative stress.

*2.2.2.2.3. Calcium regulation and the endoplasmic reticulum.* There is considerable evidence that a number of toxic chemicals target the $Ca^{2+}$ signalling processes, alter them and induce cell death by apoptosis. A perturbation of the mechanisms controlling cellular $Ca^{2+}$ homeostasis and signalling processes through exposure to drugs and environmental agents has been shown to be the basis for many diseases and other pathologic conditions such as cancer, diabetes, autoimmune diseases and neurodegeneration. The relative importance of increased calcium in cell death has been debated extensively (Farber, 1990; Nicotera et al., 1990; Reed, 1990; Harman and Maxwell, 1995; Trump and Berezesky, 1995; McConkey and Orrenius, 1997). In addition, toxic chemicals that generate oxidative stress or induce a pathologic increase in cellular calcium levels can kill their target cells either by necrosis or apoptosis, depending on the degree of exposure (Kass and Orreniun, 1998).

The ER contains a complement of stress proteins including the glucose-regulated proteins (Grps), as well as calciumbinding chaperone proteins such as calreticulin and calnexin (Helenius et al., 1997; Kaufman, 1999). Agents that disturb the ability of the ER to accumulate calcium and/or deplete ER calcium are potent signals for activation of *grp78* and *grp94*, prototypical *grp* genes (Lee, 1992; Kaufman, 1999). Grp78 [otherwise known as immunoglobulin binding protein (BiP)] and Grp94 are the ER homologs of cytosolic Hsp70 and Hsp90 and serve similar functions in protein folding.

*2.2.2.2.4. Heat shock proteins.* Exposure of all eukaryotic and prokaryotic cells to heat or a range of other metabolic stressors (including a wide range of toxicants) can result in an increase in the synthesis of one or more of a family of well-conserved proteins referred to as heat shock proteins (Hsps) (Donati et al., 1990). These proteins consist of several subgroups of varying molecular weights (i.e. Hsp25, Hsp60, Hsp70 and Hsp90), which are present in unstressed cells where they function as molecular chaperones aiding in the folding and assembly of newly formed proteins (Buchner, 1996). It has been proposed that the induction of Hsps may be utilised as markers of toxicity (Hansen et al., 1988; Pipkin et al., 1988; Gonzalez et al., 1989; Aoki et al., 1990; Deaton et al., 1990; Cochrane et al., 1991; Low-Friedrich et al., 1991). It has been observed that induction of Hsps occurs at chemical concentrations below those required for toxicity and this induction appears to be one of the initial responses of a cell following chemical exposure (Goering et al., 1993). In addition, the induction of Hsps is one of the most widely conserved responses across a range of organisms, and therefore extrapolations between species can be performed with some confidence (Boorstein et al., 1994; Rensing and Maier, 1994). Finally, the stress response of a cell is rapid and easily measurable and significant increases in Hsp levels are observed within hours of chemical exposure (Blake et al., 1990).

*2.2.2.2.5. Stress-activated protein kinases (SAPKs).* Another important aspect of the cellular response to stress-inducing agents is the induction of the stress-activated protein kinases (SAPKs). These protein kinases form a cascade, which is parallel, but distinct from the MAP kinase pathway of signal transduction. The SAPKs ultimately activate the transcriptional activator c-jun, which stimulates transcription of a wide variety of genes. Some inducers of the SAPK pathway are the same agents which induce hsp70 and immunoglobulin binding protein (BIP)/grp78 synthesis.

*2.2.2.2.6. Metallothioneins.* Metallothioneins (MT) are low molecular weight, cysteine-rich, metal-binding proteins. MT genes are readily induced by various physiologic and toxicologic stimuli. Because the cysteines in MTs are well conserved across species, it is thought that they are necessary for MT function. Results from numerous studies have indicated multiple functions of MT in cell biology including: (a) a 'storehouse' for zinc; (b) a free-radical scavenger; and (c) protection against cadmium (Cd) toxicity, which is suggested as its main function following studies with MT-transgenic and null mice. However, the induction of MT has also been demonstrated to be a cellular adaptive response (Klaassen and Liu, 1998). It affects the magnitude and progression of toxic insults from metals such as Cd and a number of organic chemicals, thereby complicating the potential use of MT induction as a marker of cell injury/death.

*2.2.2.2.7. Adaptive responses.* Most cellular responses (including the examples outlined above) are dependent on the dose of chemical and the exposure time. For example, a low dose of a particular compound for a long period of exposure may result in adaptive or even beneficial/protective effects. In addition, gene expression changes are often transient and the response time, for instance the time from exposure to chemical to detection of a change in gene expression, will vary from gene to gene. This is a particular challenge and/or limitation in considering the utilisation of cellular responses as markers of toxicity for chemicals in food, where we are more concerned about the effects following chronic exposure. Therefore, for each cellular response of interest, it is important to try to distinguish between

responses that are indicative of adverse effects, such as those that may lead to cell death or sublethal effects and those which are adaptive, protective or excitatory such as immune responses, modulation of metabolism, induction of metallothioneins and transporters.

*2.2.2.3. Conclusions and future priorities*. A considerable amount of research in the area of cellular and stress responses to chemicals and environmental agents has been conducted over the last few years and they offer much potential as in vitro screens for improved hazard identification. Indeed, reporter gene technology has already been developed and applied in screens for a number of these responses. Assays based on cellular response could involve analysis of a wide range of potential markers, with careful consideration in the choice (or the use of a battery) of relevant in vitro models. One of the major challenges is the interpretation of the results of these individual endpoints because changes in expression of these genes/proteins do not necessarily imply that the chemical will exert a toxic effect per se on long-term exposure at relevant dose levels. Therefore, one of the research needs will be to generate databases of known effects from chemicals with well-characterised toxic effects. In addition, adaptive responses of the cell could make interpretation of these changes difficult.

Careful consideration needs to be given to the choice of in vitro model used for studies investigating the cellular response to toxic chemicals. For example, information about the background levels of these potential markers and the metabolic competence of the model as compared to normal cells in vivo need to be ascertained in order to interpret the results with confidence. In addition, responses will differ depending on whether a model involving a static cell system or proliferating cells is used.

A general problem when studying stress responses is that the background levels can be elevated in in vitro systems. This is of particular importance when studying Hsps as markers of toxicity in in vitro systems. Basal stress protein levels are elevated in, for example, hepatocyte monolayers, during isolation and culture. As a consequence of this, stress protein levels in hepatocyte monolayers are not consistently elevated following chemical exposure (Dilworth and Timbrell, 1998). However, other in vitro models such as hepatocytes cultured as liver spheroids, go through a more transient period of stress, returning to basal levels after certain periods of time (Dilworth et al., 2000). Therefore, the use of Hsps as early markers of toxicity in vitro is promising, although care is needed when selecting in vitro models to use. Information should be sought on the background level for all stress response genes/proteins for each model.

Induction of many cellular stress genes/proteins is highly tissue and cell specific, due to differences in gene expression among specialized cell types. This poses a challenge in selection of suitable in vitro models in which to assess stress protein responses to toxic insult. One complication of in vitro models is that cell lines are often transformed and therefore the intrinsic properties of the cells will be different. Therefore, in vitro models need to be well characterised, for instance by comparison of transformed cell lines with tissue slices to establish any background differences in cellular profiles/responses. In addition, it is known that the in vivo response to chemical stressors is dependent on a number of factors including the distribution of the chemical among the tissues; the ability of each tissue to detoxify the toxicant and minimize cellular damage; and the chemical's molecular mechanisms of toxicity. This will be challenging to model in vitro.

Despite the challenges and limitations outlined, cellular responses could provide suitable markers for chemical toxicity (in particular providing some mechanistic understanding) and become an integral component of improved methods to assess the impact of chemicals on human health.

### *2.2.3. Perspectives for using in vitro methods to evaluate chronic toxicity of compounds*

The identification of any effects of repeated exposure to low concentrations of chemicals added to or otherwise contained in food represents one of the biggest challenges to rational hazard prediction as a basis for meaningful risk assessment. The limitations of laboratory animal studies include species differences as well as extrapolating from high dose to low dose exposure. The former is recognised de facto by the use of safety factors as a basis for deriving acceptable intake levels.

The use of non-animal test methods, including computer-based approaches and in vitro studies, also involves enormous difficulties, since their focus to date has tended to be on acute effects or on one particular type of chronic effect, namely, the induction of malignant neoplasia.

One of the possibilities for using in vitro methods for a meaningful evaluation of chronic effects would include the application of longer-term cell or tissue cultures (Pfaller et al., 2001). One general difficulty that has hampered developments of these systems is the rapid loss of tissue-specific differentiated functions in many of these cultures. An example is the loss of biotransformation enzymes in monolayer cultures of hepatocytes over the first days in primary culture (Wortelboer et al., 1991). While the exact reasons for this loss are not clear, it can be assumed that important factors in this loss of differentiated function will include the loss of interaction between different cell types, between cells and the extracellular matrix and between cells and, for example, hormonal factors. An attempt to overcome this difficulty involves the use of more complicated culture

techniques, in which (one or more of) these interactions are restored, for example hepatocytes co-cultured with other (epithelial) cells or with matrix and serum factors. Although this field is developing rapidly, no well-defined systems are presently ready to be used in hazard or risk assessments (LeCluyse et al., 2000).

Another approach is the use of cell lines in prolonged culture (Hanley et al., 1999; Pfaller et al., 2001). The effects of exposure of the cells in a continuous flow-through system can then be measured. However, in these systems only those functional parameters can be studied that are still present in these cells. While the application of cell immortalisation techniques or the construction of transgenic cells will be of use in the future further developmental work will be required (Crespi and Miller, 1999).

The considerations described in other sections of this chapter with regard to cellular responses (see section 2.2), biomarkers (see section 4), etc., will also be of value for the development of (early) markers of chronic toxicity of compounds. One example of data extrapolated from a relatively short (72 h) exposure time in vitro was the use of neuroblastoma cells to study the effect of acrylamide on the number of neurites developed by these cells. These data were the basis of a toxicodynamic model and it was shown that these data could be extrapolated over a longer period to predict the effects of acrylamide in a 90-day exposure study in vivo (DeJongh et al., 1999b).

### *2.3. Toxicokinetic modelling and metabolism*

In vitro approaches can be used to obtain useful information on the disposition (absorption, distribution, metabolism and excretion) of xenobiotic compounds. Indeed, it is in some of these areas that in vitro studies have had their greatest impact, and currently have the greatest applicability. As with all toxicology, in assessing the kinetics of a compound it is important to consider the purpose of the study. This will affect the amount and precision of the information required. Hence, kinetic studies can range from simple semi-quantitative estimates of metabolic stability to a highly detailed physiologically-based toxicokinetic model. In the section that follows, therefore, it should be kept in mind that even basic information on a single process, for example extent of absorption, can be of real value.

#### *2.3.1. Extrapolation of kinetic behaviour from the in vitro to the in vivo situation*

Toxicokinetic modelling describes the absorption, distribution, metabolism and elimination of xenobiotics as a function of dose and time within an organism. Toxicokinetic models can be divided into two main classes: data-based compartmental models and physiologically-based compartmental models (Andersen, 1991). The most useful models simulate the biological complexity of the body by their use of two or more tissue compartments.

Data-based compartmental models are the 'classical' type of models in which the body is usually represented by a system that describes uptake, distribution, including inter-compartmental exchange, and metabolism. The corresponding model parameters can be estimated by fitting the model to the data. The model parameter that describes the extent of distribution of the compound between blood or plasma and the other tissues is the apparent volume of distribution (Vd). This parameter, as well as the parameters on inter-compartmental diffusion rates, do not represent the actual tissue volumes, blood flows and diffusion rates in the body (Yang and Andersen, 1994), but are the result of all processes regarding distribution, perfusion and diffusion. Owing to the simplicity of data-based models and the limited number of parameters, a model structure can be rapidly established and parameterised on the basis of the results of in vivo studies. However, compartmental models are merely descriptive of the data, they do not allow rationalisation of the mechanisms governing the kinetic processes, and in particular an estimation of the concentration of a substance at the effect site.

The second class of toxicokinetic models, the physiologically-based toxicokinetic (PB-TK) models, describe the body in terms of series of compartments based on the known anatomy and physiology of the organism. PB-TK modelling is described in detail in section 2.3.2. Much of the chemical-specific information necessary for PB-TK modelling can be obtained by studies in vitro, including tissue–blood partition coefficients, the kinetics of any active transport processes, and the kinetics of metabolism by the liver and any other organ capable of biotransforming the compound (e.g. the lung). The blood–air partition coefficient, important in the uptake and exhalation of volatile compounds, can also be determined in vitro. One of the main advantages of a PB-TK model over a more conventional data-derived compartmental model is the ability to predict the kinetic behaviour of the compound on the basis of a mechanistically-based model structure, produced using independently derived parameters. To be of value, it is important that PB-TK models are adequately validated. As discussed in section 2.3.2., PB-TK models permit route-to-route, dose-to-dose and interspecies extrapolation, beyond the conditions of the experiment (Andersen, 1991).

Over the last 10 years, the feasibility of this modelling approach has been greatly increased due to the availability of computer techniques that allow for the simultaneous, numerical solution of differential equations (Clewell and Andersen, 1986). While many species-specific anatomical and physiological data have become available from the literature (Brown et al.,

1997), compound-specific parameters for PB-TK models, such as tissue–blood partition coefficients and the Michaelis–Menten constants $V_{max}$ and $K_m$, are often still obtained by fitting these parameters to experimental data obtained in vivo. However, as indicated below, there is considerable scope and benefit in the incorporation of in vitro-derived parameters, which will lead to a reduction of large-scale animal studies for quantitative assessment of the biological activity of xenobiotics. In addition, the effective use of PB-TK models will contribute to replacement, reduction and refinement of animal studies by enabling optimisation of study design through identification of critical parameters and timeframes in kinetic behaviour (Thomas et al., 1996).

*2.3.2. Obtaining compound-specific parameters for PB-TK modelling from in vitro studies or other non-animal models*

*2.3.2.1. Absorption.* Absorption constitutes the first step in the sequence of interactions between a compound and the organism. For compounds that are administered parenterally, the description of direct infusion into the venous blood stream is straightforward. In contrast, the absorption of compounds taken up via the food, or as a result of occupational or environmental exposure is usually oral, dermal or via inhalation. Although the biological complexity of in vivo absorption processes complicates the application of in vitro models, some techniques have been developed to estimate oral and dermal absorption processes from in vitro test systems (Kao et al., 1985; Artursson and Karlsson, 1991; Friend, 1992; de Lange et al., 1992), and this is an area that is developing rapidly. These systems consist of, for example, CACO-2 cell layers through which the compound can be transferred. Compounds are usually applied in solution in the donor compartment and can be measured in the receptor compartment (Artursson and Karlsson, 1991). Passive diffusion from the small intestine can also be estimated from a consideration of the physicochemical properties of the compound, particularly molecular weight and lipid solubility (Clark, 1999) or from simple artificial membranes. Attempts to describe dermal, as well as oral, absorption by quantitative structure–activity relationships have been undertaken (Seydel and Schaper, 1982; Austel and Kutter, 1983; McDougal et al., 1990). Often, application of these predictive systems has been limited to a series of structurally-related homologues.

For oral absorption the processes in the gastrointestinal tract may complicate the situation. These comprise not only the activity of digestive fluids produced in the oral cavity, the stomach (e.g. acid hydrolysis) or in the intestine (under the influence of pancreatic enzymes and the enzymes of the intestinal brush border), but also the activity of the gut microorganisms should be taken into account. In the last few years, an additional complication has arisen following recognition of the role played by *p*-glycoprotein and other transporters in limiting gastrointestinal uptake of some compounds (Lennernas, 1998). Many in vitro systems have been developed, both simple, such as digestion cells containing enzyme mixtures such as pancreatin, trypsin, chymotrypsin (Hertel et al., 2000) and complex, such as complicated integrated reaction systems simulating the complete GI tract. A number of in vitro models for colonic fermentation have been developed to simulate the human colon and its microbial population (McBurney and Thompson, 1989; Rumney and Rowland, 1992). While models are being developed to take account of at least some of the active processes involved in gastrointestinal absorption (e.g. Ito et al., 1999), they are at a relatively early stage and further work is required to increase understanding of the processes involved and how they inter-relate.

*2.3.2.1.1. Tissue–blood partitioning.* Tissue–air partition coefficients (PCs) of volatile compounds can be measured in vitro by incubation of the compound with a homogenate of the respective tissue in buffered saline. The equilibrium distribution of the compound is then determined by analysis of the air (Sato and Nakajima, 1979; Perbellini et al., 1985; Fiserova-Bergerova and Diaz, 1986). Tissue–blood partition coefficients can be calculated by dividing the tissue–air PCs by the respective blood–air PCs. This method has been applied in many studies on volatile hydrocarbons with both human and rat blood, liver, lung, kidney, fat, muscle and brain homogenates (Fiserova-Bergerova and Diaz, 1986; Gargas et al., 1989). This principle has been extended to tissue–blood PCs of non-volatile compounds by the use of a number of in vitro methods that are based on the incubation of compounds with a buffered tissue homogenate (Pacifici and Viani, 1992; Jepson et al., 1994). However, these techniques are time consuming and often require a radiolabelled form of the compound under consideration. All of these methods attempt to estimate the biological partitioning process as a function of non-biological data such as water and/or lipid solubility of a compound. The distribution rate of a compound to the brain may be described mathematically as a function of input into the brain from the arterial blood stream and outflow from the venous side. Assumption of a venous equilibration model implies that the concentration of a compound in the venous blood leaving the brain is always in equilibrium with that in the brain tissue. This model can be expected to be valid for small non-polar, lipophilic as well as hydrophilic compounds. Thus, the perfusion rate of the brain dominates over the diffusion rate through the blood–brain barrier as the rate-limiting step in brain distribution. Depending on chemical structure, other compounds may express a diffusion limited brain uptake in vivo, requiring a compound-specific parameter for

diffusion rate. In the case of polar and/or ionized compounds, an energy dependent, saturable uptake process, similar to the Michaelis–Menten description of metabolism, may be required to describe interaction with the blood–brain barrier. In vitro models are in development to assess these aspects of chemical behaviour (Franke et al., 2000).

Special attention should be given to the role of active transport mechanisms, such as occurring in the gut wall, the kidneys but also in the blood–brain barrier.

*2.3.2.2. Metabolism.* Metabolism is often the major process responsible for the elimination of a chemical, and hence is of key importance in the construction of a PB-TK model. However, early and accurate knowledge of the metabolic reactions involved in the disposition of a chemical can, in itself, be of considerable value for a number of reasons. These include determination of metabolic stability to obtain, for example, information on potential bioaccumulation. Identification of biologically active or chemically reactive metabolites can be extremely helpful in anticipating possible toxic reactions through either pharmacological or biochemical effects. The identification of active metabolites may also help in interpreting the dynamics of a compound. Metabolic studies aid in inter-species comparison and the interpretation of kinetic and dynamic data, particularly when trying to compare effects (or absence of effects) relative to those in humans. In addition, studies of metabolism can help in the prediction of saturability of clearance processes, the rate of clearance and the importance of different clearance pathways. Information on the enzymes involved in the metabolism of a compound can help in the prediction of the tissue and cellular targets for any effect, the extent of inter-individual variability, and whether this is likely to be due to genetic polymorphisms, environmental factors or pathophysiological effects. Finally, knowledge of the specificity of metabolism can enable a rational approach to the prediction of metabolic interactions to be undertaken and appropriate precautions to be recommended.

In the study of xenobiotic metabolism, in vitro systems are becoming more than just alternatives but the first choice in many applications. This has been driven by the dramatic expansion of our understanding of the enzymes involved over the last quarter-century, and hence the ability to develop mechanistically based systems. The need to screen large numbers of new chemical structures for their metabolic characteristics has recently reinforced efforts to establish robust in vitro systems for predicting xenobiotic metabolism. The marked inter-species differences that exist in the levels of expression and activity of many of the enzymes of xenobiotic metabolism mean that wherever possible, human-derived systems are being utilised in preference to studies in animals, either in vitro or in vivo.

A number of in vitro systems are available to study xenobiotic metabolism. These include primary cells in suspension, primary cells in culture, continuous cell lines, primary cells transformed to immortalise them, precision-cut tissue slices, subcellular fractions, such as the microsomal fraction, and cells harbouring recombinant expressed enzymes (Bäärnhielm et al., 1986; Gearhart et al., 1990; Houston and Carlile, 1997). Finally, there have been considerable advances in the development of active site models, particularly of P450 enzymes, in the last few years (Dai et al., 2000). Although, to date, predictions have been restricted to route and specificity, newer approaches involving computational chemistry may enable the prediction of rates of metabolism in the future (Segall et al., 1999).

While most of these systems are usually liver-derived, it is possible to use extrahepatic systems, for instance lung (Toftgard et al., 1986) or small intestine. For food components the role of metabolism in the gut by microbial processes or in the gut epithelium also needs to be considered. The levels of expression of xenobiotic metabolising enzymes in many tissues are now being characterised using specific reagents. This, together with the availability of a range of techniques to define enzyme specificity towards a food chemical, such as recombinant expressed enzymes, often allows prediction of routes and rates of metabolism in a variety of tissues from a relatively small number of in vitro experiments.

Special test systems have been developed for volatile organics in vitro (Hildebrand et al., 1981; Kedderis et al., 1993). Depending on the occurrence of metabolic activation and subsequent covalent binding, either the disappearance of parent compound or the formation rate of specific metabolites may be relevant for analysis of the role of kinetics in the toxicity of a compound.

Recent developments in analytical chemistry, for example in mass spectrometry, have improved the utility of in vitro systems for the determination of metabolic stability and of routes of metabolism. Estimation of disappearance of substrate at low concentrations during incubation with an appropriate, metabolically competent system provides a relatively simple and rapid means of assessing metabolic stability (Korfmacher et al., 1999). In vitro systems are well suited to the identification of routes of metabolism (Fernandez-Metzler et al., 1999). It is sometimes possible to detect short-lived metabolites in vitro that are not detectable in vivo because of their rapid further biotransformation. In addition, in vitro studies are well suited to the investigation of the mechanisms involved in the formation of metabolites. The choice of the appropriate system should be facilitated by a consideration of structure, and the likelihood of phase I and phase II metabolism, respectively. For example, compounds metabolised primarily by phase I routes are readily studied using microsomal systems. In the area of enzyme specificity, in

vitro systems offer unique advantages over all other systems. Indeed, until the availability of human-derived in vitro systems, it was almost impossible to perform meaningful studies of this type. Available approaches include correlation studies (with marker enzyme activities or specific enzyme content) using a panel of tissue samples, studies with a panel of samples genotyped for polymorphisms of xenobiotic metabolising enzymes, selective inhibition of enzyme activity (using chemical or antibody inhibitors), studies with recombinant expressed enzymes and the use of homology models of the enzymes (Andersson et al., 2001).

The recent notable success (Houston and Carlile, 1997) in relating the rate of in vitro metabolism of several compounds with the corresponding values in vivo represents a major step as traditionally the value of in vitro metabolising systems was considered to be purely qualitative in nature. The basis of this relationship is the use of the parameter, intrinsic clearance, which is a pure measure of enzyme activity towards a compound in vivo and is not influenced by other physiological determinants of clearance such as hepatic blood flow or drug binding. This parameter has an in vitro equivalent since it corresponds to the $V_{max}$:$K_m$ ratio. The utility of in vitro intrinsic clearance as a predictor of in vivo intrinsic clearance, and hence hepatic clearance and total body clearance, has been assessed with a database of 35 drug substrates for rat cytochrome P450. Others have also demonstrated the utility of the approach in predicting kinetics in humans (Obach et al., 1997; Ito et al., 1998; Lave et al., 1999). Caution should be used if biotransformation of compounds depends on enzyme systems with known polymorphisms in man. In vitro systems can take this factor into account (Diener and Gundert-Remy, 1999).

The value of in vitro to in vivo extrapolation is clearly demonstrated by considering kinetic data from incubations with freshly isolated hepatocytes. The determination of easily measurable in vitro parameters, such as $V_{max}$ and $K_m$ from initial rate studies, and scaling from the in vitro situation by accounting for the hepatocellularity, provides in vivo intrinsic clearance estimates. A scaling factor of 110 million cells per gram of liver has proven to be a robust parameter independent of laboratory technique and insensitive to animal pretreatment with various inducers and inhibitors, which alter cytochrome P450 complement and zonal distribution of isozymes. Similar procedures can also be adopted for other in vitro systems such as hepatic microsomes and liver slices. An appropriate scaling factor for microsomal studies is the microsomal recovery index, which allows for the incomplete recovery of cytochrome P450 with standard differential centrifugation of liver homogenates. This scaling factor averages 45 mg microsomsal protein per gram of liver. Using the database, microsomal prediction has proved "successful" in two-thirds of cases, while for hepatocyte data "successful" predictions have been achieved in 90% of cases. Success is defined by a predicted/observed intrinsic clearance ratio of between 0.5 and 2. The hepatocellularity of a liver slice has been a less satisfactory parameter for scaling kinetic data. The level of success varies from compound to compound and diffusion of substrate is a competing process to metabolism within the slice incubation system. Hence, low clearance compounds are better predicted than high clearance compounds. Three models describing hepatic clearance (venous equilibration, parallel tube and dispersion) have been compared to predict hepatic clearance from in vitro intrinsic clearance values. No consistent advantage of one model over others could be demonstrated. Thus, the simplest, the venous equilibration model, appears adequate for the currently available data in hepatocytes and microsomes.

Recently, a multicentre study was performed to evaluate whether and how knowledge on a compound's mechanism of toxic action and its toxicokinetics, either derived from in vitro systems or based on its structure, can form the basis of hazard identification. This so-called "ECITTS" scheme (Walum et al., 1992) is based on the idea that sets of test batteries for predicting various types of local and systemic toxicity can be combined into integrated testing schemes for making toxicity profiles for compounds. It is assumed that an "elementary analysis" of a compound's toxicity may be a more efficient approach to toxicity testing and hazard assessment, than animal-based investigations. "Elementary analysis" of toxicity would imply that the mode of toxic action of a compound can be broken down into a number of toxicokinetic and cellular events, each of which can be identified and quantified in appropriate model systems (Walum et al., 1992; DeJongh et al., 1999a).

In the ECITTS scheme the emphasis was on the neurotoxic action of compounds. Toxicokinetic models were constructed, where possible solely on the basis of in vitro-derived parameters for biotransformation as well as on partition coefficients determined or calculated from physicochemical parameters. A test battery for relevant neurotoxic endpoints was developed in one of the co-operating laboratories. Effective concentrations in the most sensitive test formed the basis of a calculation of the compound's effective dose, making use of the toxicokinetic model. This formed the basis of the prediction of a compound's systemic toxicity for acute or (sub)chronic exposure, expressed as a lowest-observed-effect level (LOEL). Both the kinetic and the dynamic models were compared with in vivo data from the published literature. While this was not an ideal data set with which to test the approach, the results of this prevalidation study did show that a good prediction of systemic toxicity could be made for the six compounds studied using different dosing regimens (acute

and subchronic). The discrepancy between estimated and experimental LOELs ranged from a factor of approximately two, for compounds with a low toxicity (e.g. a high LOEL), to a factor of 10, for compounds with a high toxicity (e.g. a low LOEL) (Blaauboer et al., 2000).

While these successes are quite encouraging since they establish the fidelity of in vitro systems for in vivo prediction, the level of success varies from compound to compound. It is important to address the reasons for failure of prediction by each in vitro system and it is noteworthy that the current approach simplifies several key issues. Three potentially important issues have been identified that need to be addressed in applying an in vitro approach to either PB-TK or metabolic stability screening. These are:

a) Assessment of the importance of sigmoidal enzyme kinetics on the determination of intrinsic clearance.
b) Assessment of the use of substrate depletion as an alternative to $V_{max}$:$K_m$ determination for compounds where metabolic fate has not been extensively characterised. Questions arising in the use of this approach include: which sample should be analysed (whole incubate, separate cells or media), the effect of media binding and the impact of prolonged metabolite production.
c) The role of product inhibition in hepatic microsomes due to non-functional phase-II metabolism.

In addition to in vitro to in vivo extrapolations, some attempts have been made to correlate the metabolic rate constants of a series of halogenated methanes and ethanes with their chemical structure (Gargas et al., 1988). Reasonable correlations were obtained for compounds that are exclusively metabolised by a particular isoform of the cytochrome P450 superfamily. However, the predictive power of such techniques should be interpreted with caution, since the mechanistic significance of molecular descriptors for the metabolic process may be very dependent on the specific chemical structure of the homologues that are studied, and the enzyme/isoform(s) involved in their metabolism. Considerably more studies are required before predictions from chemical structure possess the requisite reliability.

## 3. The use of in vitro methods in strategies for characterising and predicting hazards to the human

In this section a wide spectrum of in vitro and other non-animal approaches is discussed that can now, or in the foreseeable future, be of use in the hazard and risk assessment of compounds present in food. Some of these methodologies will have their place in procedures as a stand-alone method, for example a mutagenicity test with a bacterial system may give an answer to the hazard of a compound to be a mutagen. However, it is clear that the potential of these approaches in toxicological risk assessment has yet to be fully realised. For instance, the addition of new chemicals to cultured cells derived from a particular tissue enables the determination of the concentration of that chemical at which a certain effect occurs in that cell type, and although it sometimes leads to a knowledge of the fundamental mechanisms underlying the toxic effects, it does not yield the profile of toxic effects caused by the chemical in the intact organism. Alternatively, a number of studies have attempted to combine the use of a variety of methods (Basketter et al., 1995; Barratt et al., 1996). Typically, the methods and data are combined in parallel or in series, and are applied before any animal tests are carried out. Studies such as these have given rise to the concept of extrapolating from in vitro to in vivo using the parallelogram approach or an "integrated testing strategy".

### 3.1. Parallelogram approach

In vitro toxicology methods can have an important function in predicting risks of chemicals for humans by enhancing the possibilities of extrapolating data derived from animal studies. This is exemplified in the so-called parallelogram approach (Blaauboer et al., 1990). In this approach, the data derived from a study with animal cells can be compared with the animal-derived data (Fig. 1). These comparisons should take into account the differences between the in vivo and in vitro situations (see section 2.3, The use of toxicokinetic data in linking the in vitro toxic concentration to the toxic dose in vivo). In parallel with this, the application of human-derived in vitro systems enables another comparison: for example, between the animal cellular system and the human cell system (Pool-Zobel et al., 1994; Pool-Zobel and Leucht, 1997). Both comparisons can then be used

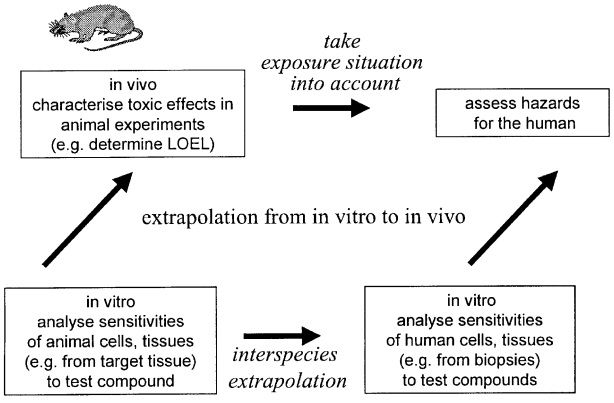


Fig. 1. General scheme of parallelogram approach to characterise hazards, including extrapolation of effects from animal species to the human and from the in vitro situation to in vivo.

to extrapolate to the situation in humans (Sobels, 1980). This strategy, supported by in vivo, biomarker-based analysis in animals and humans, is the way forward to adequately assess hazards within a parallelogram approach. It allows quantitative, mechanism-driven interspecies evaluation, necessary for the plausible extrapolation from the in vitro to the in vivo situation and from animals to humans. Cross-linking to genomics, transcriptomics and proteomics approaches will enhance relevance of hazard characterisation and will increase the power of predictability.

### 3.2. Integrated test strategy

"An integrated testing strategy is any approach to the evaluation of toxicity which is based on the use of two or more of the following: physicochemical, in vitro, human (e.g. epidemiological, clinical case reports), animal data (as available or if unavoidable), computational methods, such as (quantitative) structure–activity relationships ([Q]SAR), structural alerts and toxicokinetic models" (definition taken from Blaauboer et al., 1999).

Relevant questions to be asked in such an integrated approach will be:

- what do we need to know;
- when do we need to know this;
- what is the most appropriate method to obtain the knowledge, also taking into account the wish to avoid animal experimentation;
- what will we do with the knowledge obtained.

Making use of the result of the ECITTS programme (DeJongh et al., 1999a; Blaauboer et al., 2000), a more generic scheme for the evaluation of a compound's local and/or systemic toxicity has been proposed (Blaauboer et al., 1999). A prerequisite for the success of such a scheme is the availability of "fit for purpose" in vitro methods. A key feature is the presence of decision points, such as stages at which decisions are made with regard to further testing. The important questions listed above will have to be asked at each branch point in the scheme and transparent criteria for decision making will have to be applied. In addition, the scheme allows for expert judgement to be made at different stages of the scheme for the purposes of hazard assessment (including classification and labelling). The ideal scheme, completely depending on non-animal methods, consists of a number of steps, including:

1. Assessments based on physicochemical properties and chemical functionality and application of [Q]SARs;
2. Toxicokinetic modelling, including the modelling of biotransformation, tissue partition and transport, together with basal cytotoxicity testing;
3. The selection from a battery of specific cytotoxicity and cell-specific function toxicity tests in the case of high tissue concentrations; and
4. The selection from a battery of specific cytotoxicity tests, if indicated by historical data for related chemical structures, regulatory requirements, or by the intended usage of the chemical.

On the basis of the outcome of each step, expert judgement would result in identification of toxic hazard and/or progression to the next step of the scheme. Thus, the scheme can be used both for screening purposes and for generating more comprehensive toxicological profiles. It is also not necessary to follow the scheme from top to bottom. Depending on the questions to be asked (e.g. what do we need to know), some parts of such a scheme may be by-passed.

If we now attempt to implement the strategies described above in the hazard and risk assessment of food constituents, it will be clear that the "ideal" situation, where it is possible to perform a full risk assessment on the basis of this scheme, has yet to be reached. Nevertheless, the general framework of the integrated use of data from different sources is equally important in each strategy (see also Barlow et al., 2002).

One important extension to the scheme would be to consider the exposure to a compound. For instance, if the compound under study is present in food only in very low concentrations (e.g. a fragrance), the concept of "Threshold of Toxicological Concern" (TTC) could be applied (Kroes et al., 2000). The TTC concept is discussed in more detail in Edler et al., 2002). Here too, however, it is important to examine the compound's structure to determine whether there are chemical functional groups of concern (structural alerts).

Another general consideration with the scheme is that there can be input from existing animal studies. As an example, quantitative structure–activity relations (QSARs) can be made on the basis of in vivo studies. Such QSARs should be used where appropriate. Furthermore, it should be acknowledged that for a number of biological endpoints, non-animal studies are not (yet) available, for example in allergic reactions. Thus, the logical integrated scheme will make use of elements from different sources: exposure, structure, physico-chemical properties, physiologically based models for kinetics and dynamics, in vitro studies, in vivo studies. A general outline of a generic scheme is described below, taking into account the above mentioned considerations, for example exposure, availability of toxicological data, etc.

#### 3.2.1. Anticipated exposure levels

Initially, the question "what do we really want to know" depends principally on the way in which exposure occurs as well as levels of exposure. For com-

pounds in food, important factors such as the amount present in food, the matrix, the duration and the frequency of exposure, will be major determinants of the knowledge needed for a risk assessment. At this step the concept of TTC may play a role. However, if there are structural alerts for certain toxicological effects, such as mutagenicity, the concept would set the threshold for the exposure levels at very low levels.

*3.2.2. Existing toxicological knowledge*

The next step takes into account the existing literature on the toxicity of a compound. This may lead to the conclusion that no further testing is necessary and that on the basis of these (animal and non-animal) data, a realistic risk characterisation can be made.

*3.2.3. Application of data on physicochemical properties*

In the next step, chemical structure and any relevant physicochemical data (e.g. melting point) are taken into account. Some physico-chemical parameters (e.g. log P) and chemical functionality (e.g. electrophilicity) can be determined from the chemical structure. Using appropriate QSAR models, these data are utilised to predict toxicological properties. Currently available models include those for the prediction of skin irritation and corrosivity (Barratt, 1996; Barratt et al., 1996), eye irritation (Barratt, 1997), skin sensitisation (Cronin and Basketter, 1994) and mutagenicity (Benigni et al., 1994), for specific classes of chemicals.

*3.2.4. Toxicokinetics*

Central to this part of the scheme is information on the kinetics of the chemical. In some situations it may be sufficient to obtain relatively basic information such as fraction absorbed, whereas in other situations advanced information such as complex toxicokinetic models will be required. PB-TK models describe the time course of the concentration of a chemical in various tissues of the body. Models can be constructed for any animal species of interest, provided the appropriate physiological parameters are available (e.g. blood flows, tissue size and composition). A comprehensive compilation of these parameters is provided by Brown et al. (1994). Compound-specific parameters, such as tissue partition coefficients, can be calculated from physico-chemical properties, or can be measured, whereas parameters such as the rates of biotransformation and transport need to be determined with appropriate in vitro systems (Arturrson and Borchardt, 1997). When bioactivation is identified as an important process, the toxicokinetics of any relevant metabolites also need to be incorporated. The integration of physico-chemical properties and in vitro data into physiologically based toxicokinetic models is illustrated by DeJongh et al. (1997, 1998) and by Houston and Carlile (1997).

An important element in toxicokinetics is the determination of a compound's biotransformation, especially if this would involve the formation of bioactivated metabolites. Many in vitro systems are already in use to determine the metabolism, mostly based on hepatocyte cultures or on cell lines in which certain (human) biotransformation enzymes have been transgenically expressed.

*3.2.5. Basal cytotoxicity*

The following step is the incorporation of knowledge about the basal cytotoxicity of a chemical and any relevant metabolites, for instance toxicity towards processes, which are common to all cell types (Seibert et al., 1996). This can be determined using one of a number of basal cytotoxicity tests, such as the neutral red uptake (NRU) assay.

The output of toxicokinetic models is the prediction of concentration/time courses in different tissues. This information can be combined with the basal cytotoxicity data to make a prediction of the acute systemic toxicity of the chemical. Thus, chemicals that are potentially hazardous to specific tissues can be identified. It may also be possible to predict non-specific effects, such as the accumulation of non-reactive lipophilic chemicals in biological membranes; this phenomenon is referred to as baseline toxicity, and manifests itself as a general "narcotic" effect in the nervous system (DeJongh et al., 1998).

*3.2.6. Specific toxicity*

If structural characteristics of the compound under study give rise to concern about specific effects in certain tissues, or if the outcome of toxicokinetic models indicates the presence of high concentrations of a chemical in a particular tissue, then the toxicity of the chemical in that tissue needs to be determined. Two forms of specific toxicity are recognised:

a) Selective toxicity, in which some cell types are more sensitive than others; and
b) Toxicity based on impairment of functions specific for certain cell types ("cell-specific function toxicity"). This could include effects that are critical to the organism as a whole, such as the inhibition of albumin production in hepatocytes, cytokine production or hormone production (Seibert et al., 1996).

The outcome of the experiments of this type can be expressed as the ratio of the effective concentration for basal cytotoxicity to the effective concentration for the cell-specific toxicity. Ratios considerably greater than one would indicate specific target organ toxicity, and a requirement to determine a "no observed effect level" (NOEL). The choice of the cell types is determined on

the basis of toxicokinetic considerations and on the presence of the potential targets to toxicity in the cell.

This part of the scheme obviously relies on the availability of well-evaluated in vitro methods, and therefore, if these are not available, in order to answer the question "what do we really need to know" it may be necessary to carry out animal experiments, according to OECD guidelines.

#### *3.2.7. Specific requirements*

Even when a chemical does not appear to accumulate in any specific tissue, or when its ratio of basal cytotoxicity to specific toxicity is not particularly high, there may still be other reasons for investigating more closely the tissue-specific effects of the chemical, namely to comply with particular regulatory requirements related to the expected usage of the chemical. In such cases, test systems could include tissue-specific tests similar to those used in earlier steps of the scheme, but they could also include tests for other toxicological endpoints, such as developmental toxicity and carcinogenicity. The outcome of this stage may also result in the classification and prediction of systemic toxicity of the chemical.

If a chemical cannot be evaluated toxicologically by applying these four stages, it may be necessary to conduct limited in vivo studies. However, the information gained in previous stages will enable such studies to be more focused than they would have otherwise been, so they will indeed be limited. Thus, these in vivo studies can be regarded as true refinements in comparison with animal procedures based on current testing guidelines.

## 4. Endpoints of in vitro toxicology systems

In the following section, a selection of different endpoints detectable by using in vitro systems is presented. They are the ones most widely used as the determination of genotoxicity for identifying potential carcinogens. In this context, however, it must be recognised that each of the three levels of mutation, namely gene, chromosome and genomic mutations (i.e. numerical chromosome changes leading to aneuploidy) may play a role not only in cancer, but also in inherited disorders and congenital defects.

### *4.1. Cancer-related endpoints*

#### *4.1.1. Introduction*

The separation of carcinogens into those that induce tumours by a genotoxic mechanism and those that are non-genotoxic can be helpful in the risk assessment process (Roe, 1998). Genotoxic carcinogens can be defined as those that directly affect DNA, either by themselves or through a metabolite (Pitot, 1993). In contrast, non-genotoxic carcinogens act by a mechanism that does not involve any direct effect on DNA (Hayashi, 1992). This distinction is of profound importance in developing short-term tests (Clayson and Iverson, 1996). Genotoxic carcinogens act through a chemical interaction with DNA and hence can be detected in vitro, at least in theory, using relatively simple test systems (Kramer, 1998). The successes of tests such as the Ames/*Salmonella* test demonstrate that this is so (Ashby and Tennant, 1991). In contrast, non-genotoxic carcinogens have no direct interaction with DNA; they cause tumours through an indirect mechanism involving pathological or physiological changes and as a consequence are not readily amenable to detection by current in vitro test systems (Ames and Gold, 1997). There can be considerable specificity in the effects of non-genotoxic carcinogens (Williams and Whynser, 1996), whereas genotoxic carcinogens will invariably be able to damage the DNA when they reach the target (Brusick, 1986).

It is now widely accepted that the vast majority of genotoxic carcinogens can be detected with some confidence using the recommended battery of in vitro and in vivo short-term tests for genotoxicity (Ashby, 1991), although refinements are possible, as discussed below. In marked contrast, there are no generally accepted methods for identifying non-genotoxic carcinogens. In view of the plethora of mechanisms involved, it would seem impossible that a single in vitro test could ever serve this function (Yamasaki et al., 1996). In considering the development of possible tests, it might be helpful to review the mechanisms involved.

Cancer can be described as a disease in which a certain cell type multiplies without apparent control, and may come about either because of an increased rate of cell division, or a decreased rate of cell death (e.g. apoptosis), or possibly a combination of both. Tumor cells exhibit many phenotypic differences from their normal counterparts, which is a consequence of a change in genotype involving usually oncogenes, tumor suppressor genes and DNA-repair genes (Barrett and Ts'o, 1978). These may show mutation, deletion or rearrangement. In general, non-genotoxic carcinogens are believed to act by inducing effects which favour the selection of cells which have been or become initiated due either to spontaneous errors or to induced damage by secondary mediators such as reactive oxygen species (Ames and Gold, 1997). Hence, many non-genotoxic carcinogens ultimately act via a genotoxic species (Klein and Klein, 1984).

#### *4.1.2. Genotoxicity*

*4.1.2.1. Introduction.* It is acknowledged that the generally accepted objectives of genotoxicity testing of chemicals are: (a) identification of germ cell mutagens, because of their possible involvement in the etiology of human heritable genetic defects; (b) identification of

somatic cell mutagens, because of their involvement in neoplastic transformation. Some of the test guidelines may be also applied in assessing the effects of genotoxic chemicals on environmental compartments or ecosystems (geno-ecotoxicology). Beyond playing a key role in chemical hazard identification, genotoxicity testing can also provide information on mechanism of action, which is pivotal in the characterization of carcinogenic risk, supporting the use of non-threshold models for the estimation of low dose effects.

It is recognised that each of the three levels of mutation, namely *gene, chromosome* and *genome mutations* (i.e. numerical chromosome changes leading to aneuploidy) may play a role in inherited disorders and cancer.

For the latter in particular, extensive analysis of mutational spectra in human tumours demonstrate the *activation of proto-oncogens* through point mutation, translocations, inversions, and/or the *inactivation of tumour suppressor genes* by loss of heterozygosity or other mutational events (Vainio et al., 1992), such as mutations in DNA repair genes, which can trigger the accumulation of genetic alterations in cancer related genes (Loeb and Loeb, 2000). Other modifications of the genetic material (e.g. recombination, amplification, hypomethylation, etc.) are suspected to play a role as well.

Aneuploidy makes a major contribution to human embryonic loss and some birth defects such as Down's syndrome. A large number of chemicals are known to induce aneuploidy in vitro or in vivo, in most cases by modifying the spindle of the dividing cells (Aardema et al., 1998). A large number of aneugens are also inducers of malignant transformation in Syrian hamster cells in vitro (Gibson et al., 1995).

*4.1.2.2. State of the art*. Analysis of a large database of genotoxicity testing programmes (Benigni, 1992; Zeiger, 1994) has shown that in general increasing the number of tests in a standard battery does not necessarily increase its sensitivity in predicting carcinogenesis. This evidence permits the use of reduced batteries of assays, based on their operational complementarity.

Owing to the variety of molecular lesions potentially relevant in cancer aetiology and in transmissible genetic defects, the analysis of *multiple genetic endpoints* is generally regarded as necessary in screening of chemicals for their genotoxicity. At present, no single validated test method can provide information on all the above-mentioned genetic endpoints: consequently, it is necessary to test each chemical in multiple assays to obtain full information on its genotoxic potential. In general, at least two in vitro short-term tests, one measuring the ability of the substance to induce mutations at the gene level in bacterial cells, and one at the chromosomal level in mammalian cells, are considered necessary in case of chemicals of limited or no human exposure (Ashby, 1986). In the case of chemicals for which extensive and/or direct human exposure is expected (e.g. food additives, pharmaceuticals), several testing strategies have been proposed over the years which comprise three to four tests at the gene and at the chromosome level, often also including an in vivo assay (FDA, 1982; Carere et al., 1995; Muller et al., 1999; Committee on Mutagenicity of Chemicals in Food, 2000). Actually, the need for an in vivo assay in a standard battery of tests for screening purposes is not fully supported by current experience. The inspection of large genotoxicity databases in fact indicated that chemicals uniquely or prevalently positive in vivo may be very rare. Thus far, only few examples have been reported in the literature, for example benzene, procarbazine, 1,2-dimethylhydrazine, urethane (Muller et al., 1999). These chemicals are believed to be bioactivated in vivo through pathways not reproduced by standard activating systems used in in vitro assays, for example involving extrahepatic metabolism or P450 enzymes poorly represented in rat liver S9 fraction.

Several mutagens and carcinogens express their activity only after metabolism. Standard in vitro tests involving the presence of a metabolic activating system in general use rat hepatic subcellular fractions, for that purpose. However, the predictivity for the human needs further improvement. In that respect, cell lines stably expressing human P450 cDNA are used as models to investigate pro-mutagens and pro-carcinogens (Kappers et al., 2000). Metabolising systems directly derived from human tissues (e.g. biopsies) are increasingly used to enhance predictivity.

*4.1.2.3. Testing strategy*. On the basis of the previous considerations and also taking into account the international recommendations for the reduction of the use of laboratory animals (EC, 1986), the following standard battery of three in vitro genotoxicity tests is, in general, recommended for low molecular weight chemicals:

1. a test for induction of gene mutations in bacteria;
2. a test for induction of gene mutations in mammalian cells (preferentially the mouse lymphoma *tk* assay);
3. a test for induction of chromosomal aberrations in mammalian cells.

In general, this battery is considered as the core set for such compounds although there may be circumstances under which it may be justified to deviate from the core set. In such cases a scientific justification is needed. A reduced battery (one or two in vitro tests), or even no testing, could, for example, be acceptable in cases where considerations on physico-

chemical, structural and metabolic properties would indicate lack of genotoxic activity.

On the other hand, supplementary modified conventional in vitro tests or other in vitro unconventional test methods may be required, in the case of in vivo structural alerts for genotoxic activity, suspected aneugens, or when pharmacokinetic/metabolic data indicate that in vitro tests are of limited significance (e.g. need of extrahepatic metabolism or P450 enzymes poorly represented in liver S9 fraction).

Special considerations should be used for novel food ingredients, including those derived from biotechnology/genetic engineering (e.g. enzyme preparations derived from microorganisms, recombinant proteins, etc.), for which the interaction with DNA or other chromosomal material is unlikely. In these cases, testing strategy (reduced battery or even no testing) should be decided on a case-by-case basis, as suggested for pharmaceutical drugs (CEC, 1989; Gocke et al., 1999). When needed, the bacterial gene mutation test can be used for detecting genotoxic impurities/process contaminants.

*4.1.2.4. Test methods*. As far as test methods are concerned, it is recommended that OECD protocols be used. In 1997 the OECD (see Barlow et al., 2002) updated six previous guidelines (Nos 471, 473, 474, 475, 476, 483) and introduced a new one (No. 486). These guidelines provide guidance for the conduct of in vitro screening tests (e.g. gene mutations in bacteria and in mammalian cells, chromosomal aberrations in vitro) as well as for the in vivo assays (e.g. micronuclei and chromosomal aberrations in rodent bone marrow, rat liver unscheduled DNA synthesis, chromosomal aberrations in spermatogonia). The same updated test procedures are going to be adopted as official test methods by the European Union.

Moreover, a protocol for the in vitro micronucleus test is currently being evaluated for inclusion in OECD guidelines for genetic toxicology testing. In the near future, this test procedure might be considered in test batteries as an alternative to in vitro chromosomal aberration assay, being able to detect both clastogens and aneugens.

Methods to detect aneuploidy have been developed using fluorescence in situ hybridization (FISH). By using centromeric specific probes, chromosome loss and non-disjunction specific probes allow rapid scoring of aneuploidy in a variety of cell types, including human cells.

Currently, mammalian cell assays should be routinely performed according to the standard updated protocols for the detection of either gene mutation (at *tk*, HPRT, or other loci), or structural chromosomal aberrations by metaphase analysis.

Following a positive result in vitro, further testing in vivo is normally required to ascertain whether the compound is also active in the whole animal (see Barlow et al., 2002).

*4.1.2.5. Novel developments*. Considerable research in many biomedical fields is directed at detecting DNA damage and mutations in specific genes. These approaches may serve as the basis to further develop in vitro systems for investigating the interaction of food compounds with the genome. In this area a number of points need to be considered:

1. **Cells and tissues**: The future genotoxicity assays should focus on using human cells from relevant tissues. These tissues are targets for disease induction by food-related compounds. For example, if colon, breast or stomach cancers are strongly associated with dietary factors, then cells from these tissues are probably best suited to investigate unknown hazards by food compounds in a mechanisms derived approach. Tissues, or cells isolated from human tumours and immortalized and primary cells from normal tissue and are being used as targets.
2. **Endpoints**: Mutations in those target genes involved in the genesis of the disease or relevant for elucidating mechanisms can be investigated with new techniques, such as those also used to disclose unknown polymorphisms (see section 2.2). Moreover, a number of useful techniques are being developed with which it is also possible to determine DNA damage in interphase DNA. The ''single cell microgel-electrophoresis assay'' (also known as Comet test), is one of these promising developments, with which specific types of damage (e.g. oxidized or otherwise damage DNA bases), or damage in specific genes (Comet FISH) can be determined by using different test protocols. Furthermore, by measuring the persistence of damage, it is possible to obtain first information relating to DNA repair kinetics. Methods for detecting point mutations based mainly on PCR technology are at various stages of development and validation; one recently improved method is the restriction site mutation assay (RSM) (Jenkins et al., 1999). In principle, this technique allows detection of mutations induced by chemicals and spontaneous endogenous process in ubiquitous restriction enzyme sites and is, therefore, readily applicable to any sequenced gene and/or cell/organism, including human cells and tissues. In addition to identifying potential mutagenic agents, the RSM assay can provide useful data on mutational events involved in carcinogenesis, for example to screen for p53 hotspot mutations, where they fall in suitable restriction enzyme sites.

*4.1.3. Non-genotoxic cancer endpoints*

*4.1.3.1. State of the art*. Non-genotoxic carcinogens can be divided into compounds that act by the following mechanisms:

*4.1.3.1.1. Persistent cytotoxicity accompanied by proliferative regeneration.*. Many carcinogens are believed to act through this mechanism (Butterworth et al., 1992), particularly when tested at MTD (Ames and Gold, 1992), which by definition will produce some toxicity. Other compounds cause cytotoxicity at a specific site, often due to toxicokinetic factors, leading to tumours at doses less than the MTD. Examples include chloroform (IPCS, 1994), alcohol (IARC, 1988), physical agents causing local irritation such as high salt in the stomach (Coggon et al., 1989) or crystalline deposits in the bladder (e.g. 2-phenylphenolate sodium) (IARC, 1987). Not all cytotoxicants are carcinogens (Butterworth and Bogdanffy, 1999). It appears that for carcinogenicity to develop, cytotoxicity needs to be persistent, so that there is continuing regeneration. Furthermore, proliferating cells might be less sensitive to the cytotoxic agent, possibly because of reduced activity of some activating enzymes (Marie et al., 1988). Often, cytotoxic agents generate ROS, which can interact with DNA causing genotoxic effects (Ames and Gold, 1997).

*4.1.3.1.2. Chronic inflammation*. A number of compounds can directly or indirectly activate inflammatory cells such as neutrophils and macrophages with the production of reactive oxygen species and possibly other free radicals (Martin et al., 1997). These may interact with DNA and so initiate tumour development (Klaunig et al., 1998). There are numerous examples of carcinogens that act by this mechanism (Feig et al., 1994), and it is likely that it is the mode of action of several other carcinogens (mechanisms of action presently unknown) or contributes to their effects. Examples include captan (Reuber, 1989) in the small intestine, asbestos and other inhaled fibres in the lung (Shi et al., 1998; Gulumian, 1999), particulates in cigarette smoke (McCusker, 1992) and possibly ozone (Cho et al., 1999) in the lung.

*4.1.3.1.3. Hormones*. Carcinogens may act either as hormone-mimetic agents or may perturb the balance of endogenous hormones. Many hormones are mitogenic (Preston-Martin et al., 1990) and hence persistent agonism can lead to cell proliferation and the probability of transformation to malignancy (Henderson et al., 1991; Castro et al., 1993). Examples of direct-acting agents in this category include synthetic estrogens (IARC, 1987) and possibly some dietary isoflavanoids (Whitten and Naftolin, 1998), while examples of the latter group include agents which induce the metabolism of thyroid hormone leading to an up-regulation of thyroid stimulating hormone (McClain, 1989), and those that inhibit gastric acid secretion leading to increased gastrin levels (Karnes and Walsh, 1990).

*4.1.3.1.4. Ligands for xenobiotic induction receptors*. It has now been clearly established that in rodents several classes of monooxygenase inducers, including peroxisomal proliferators, phenobarbitone-like inducers and dioxin-like inducers, can all stimulate a pleiotropic response in liver (Farber, 1990), subsequent to interaction with their complementary receptor (Waxman, 1999) leading to, among other effects, an increase in the rate of hepatocyte division and inhibition of apoptosis (Hasmall and Roberts, 1999). This often leads to hepatocellular carcinoma in rodents (Marsman and Barrett, 1994). The receptors involved are the Ah receptor (AhR) for dioxin and related compounds, CAR for phenobarbitone and related compounds and PPARα for peroxisomal proliferators (Waxman, 1999). While ligands for the first two receptors lead to an induction response in non-rodents, as in rodents, this does not appear to be associated with mitogenesis (James and Roberts, 1996), the reason for this being as yet unknown. Dioxin and related compounds can cause tumors of tissues other than the liver, which also appear to be dependent on interaction with the Ah receptor (McGregor et al., 1998). However, not all inducers acting via these receptors are necessarily carcinogenic, even in rodents (Anderson et al., 1990) and this may be a reflection of potency and persistence of response.

The receptor, following binding of an appropriate ligand, dimerises with a transcription partner, in the case of AhR this is ARNT and in the case of CAR and PPARα, it is RXR. The dimer then binds to recognition elements upstream of responsive genes leading to their transcriptional activation (Waxman, 1999). The targets leading to tumorigenesis have yet to be identified.

*4.1.3.1.5. DNA methylation*. DNA transcription can be regulated by methylation at specific CpG sites. Such methylation is reversible and is controlled by specific enzymes. However, there is increasing evidence that dysregulation of cytosine methylation (either increased or decreased) is involved in some cancers induced by chemicals (Minamoto et al., 1999). The DNA methylation pattern at CpG sites in the promoter region of certain genes affects the level of mRNA and hence protein expression. Changes in methylation can be induced by a variety of non-genotoxic mechanisms, for example by altering signalling pathways (Wachsman, 1997).

*4.1.3.2. Limitations*. Many non-genotoxic carcinogens are species, tissue and often sex or strain specific. It is now widely accepted that some of the mechanisms involved are such that compounds acting in this way

pose minimal risk to humans (Williams and Whynser, 1996). Examples include peroxisomal proliferator-induced rodent hepatocarcinogenicity (Bentley et al., 1993), thyroid tumours arising from induction of UDP-glucuronysyltransferases (McClain, 1989) and renal tumours arising from the interaction of *d*-limonene with alpha$_{2\mu}$-globulin in male rats (Hard et al., 1993). Hence, in considering the design of short-term tests for non-genotoxic carcinogens, the focus should be on mechanisms and endpoints of relevance to human risk. In most cases, compounds causing cancer in humans by a non-genotoxic mechanism appear to do so only at high levels of exposure, such as occupationally or during therapeutic administration. Among the likely non-genotoxic carcinogens most act either as hormonal agonists (proestrogens) or provoke a persistent inflammatory reaction, for instance in the respiratory tract (e.g. inhaled particulates). The remainder induce regenerative proliferation as a consequence of chronic cytotoxicity or irritation, apart from cyclosporin and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). The carcinogenicity of cyclosporine may be related to its immunosuppressive properties (Hojo et al., 1999), while TCDD may act via the Ah receptor (IARC, 1997). Among some genotoxic carcinogens, such as shale oils (Clark et al., 1988) and tobacco smoke (McCusker, 1992), there is evidence for local irritation and inflammation which may be related, at least in part, to their tumorigenicity. Hence, such compounds might be expected to be positive in appropriately designed tests for non-genotoxic carcinogens.

*4.1.3.3. Novel developments*. Design of tests for non-genotoxic carcinogens Based on the foregoing, the following points need to be considered in the design of any potential short-term test for non-genotoxic carcinogens.

1. A number of the mechanisms whereby non-genotoxic carcinogens give rise to tumours in experimental animals are of minimal relevance in human risk assessment.
2. Mechanisms that appear to be relevant for human non-genotoxic carcinogenicity are persistent inflammation in the respiratory tract, chronic irritation, primarily at point of contact and hormonal (mainly estrogenic) agonism.
3. There are many potential targets and initiating mechanisms (e.g. growth factors) involved in non-genotoxic carcinogenicity. Hence, it would not be helpful to try to develop a general in vitro test that might detect all non-genotoxic carcinogens, in the way that there are general tests for genotoxic carcinogens.
4. Much is still not known about the basic biology of chemical carcinogenesis, particularly that induced by non-genotoxic agents. While the initial events are often identifiable, the factors, which result in progression to malignancy and which dictate species or strain specificity, are not always known.
5. The final common pathway appears to involve increased rates of cell division and/or decreased cell attrition via apoptosis (of initiated cells).
6. The eventual consequence of the initial biological effects of potentially non-genotoxic carcinogens is dependent on a number of modifying factors, including persistence, resistance, the amount of spare capacity in homeostatic mechanisms and genetic susceptibility factors. The latter play a marked role in determining strain differences in the effects of non-genotoxic carcinogens.

With these considerations in mind, several possible avenues leading to the development of short-term tests for non-genotoxic carcinogens can be envisaged

*4.1.3.3.1. Development of in vitro systems to detect compounds acting by the major mechanisms involved in non-genotoxic carcinogenesis. Detection of mitogenesis* — As a common observation in the mechanism of action of many non-genotoxic carcinogens, is an early stimulation of cell proliferation, methods for the most effective way of screening a large number of tissues and cell types for increased turnover need to be developed. Ideally, these would avoid the need to administer a reporter molecule such as bromodeoxyuridine (Elias, 1997). Current measures of cell proliferation such as AgNORs (argyrophilic nucleolar organizer regions), PCNA (proliferating cell nuclear antigen) and the expression of other cell cycle proteins such as cell cyclins (Elias, 1997) should be compared with other more sensitive measures of cell proliferation, possibly utilising in situ hybridisation or RT-PCR.

*The application of gene arrays and other approaches to transcription profiling* — As indicated above, there is a need to identify sensitive early markers of cell proliferation and other key events in the induction of malignant transformation such as apoptosis and progression. The increasing availability of gene arrays from a number of species will help establish which other pivotal early events are involved in triggering a tumorigenic response (Rockett and Dix, 1999; Afshari et al., 1999). It should be noted that while many compounds are cytotoxic, only some produce tumours and hence it is clear that modulating downstream events play an important role in determining carcinogenic potential. The application of gene array technology and proteomics to an investigation of the events occurring at different stages of tumour development may be extremely informative here, in helping identify the important endpoints (Kondoh et al., 1999).

*In vitro cell transformation assays* — There is some interest in the possibility of using such systems to identify

non-genotoxic carcinogens (Yamasaki et al., 1996). An example of such a system is the Syrian hamster embryo (SHE) cell transformation system (Nguyen-Ba. and Vasseur, 1999). However, these would respond only to a limited range of compounds and would be unlikely to detect the majority of human carcinogens acting by a non-genotoxic mechanism. Nevertheless, identification of a group of compounds in this category would be invaluable in any study in which a cell transformation system was being evaluated. Other in vitro systems that have been under development for some time include inhibition of gap junctional intercellular communication (GJIC) (Yamasaki et al., 1996; Combes et al., 1999). The validity of such systems has been the subject of a recent IARC publication (IARC, 1999).

*Transgenic cell systems* — There is potential in the development of specific, genetically engineered cell systems for detecting certain classes of non-genotoxic carcinogens. This would need a significant investment of research effort both into basic biology and into constructing suitable cell lines. One possibility would be to create a reporter construct that can be activated in any cell undergoing proliferation, amplifying the often low signals presently observed. The readout of such a system could either be histological or biochemical through the release of a soluble factor into the medium. Other areas that might benefit from the development of a transgenic model would be the ready detection of persistent inflammation and an increase in endocrine activity, such as to estrogens. In this last instance, considerable research effort is already been expended in these areas, for other reasons (Nishikawa et al., 1999). It might be possible to take advantage of the results of some this work, in developing new models for detecting 'hormonal' carcinogens.

*Cytosine methylation* — Many of the current approaches to the detection of methylation patterns of DNA are cumbersome and time consuming, but recent developments, such as the MethylLight system (Eads et al., 2000), should make it feasible to develop in vitro assays to assess the effect of chemicals on DNA methylation status. However, as for some of the other endpoints discussed here, the choice of cell type will be critical, to ensure that the event proximal to changes in methylation can be reproduced in the test system.

*Quantitative structure–activity relationships (QSAR) and other computational approaches* — Many groups are investigating the possibility of developing QSAR methods for the prediction of the toxicological properties of chemicals, some of which may be of relevance to non-genotoxic carcinogenicity. Examples include estrogenic potency, AhR activation and irritant potential. Eventually, some of these may be sufficiently reliable to provide an early screen for non-genotoxic carcinogens acting via specific mechanisms. Research in this area should not be ignored, particularly when allied to physiologically-based toxicokinetics, to provide some estimate of likely target exposure in vivo (Yang et al., 1998).

*4.1.3.4. Conclusion.* No single system will be adequate to detect all non-genotoxic carcinogens or even a large number of them. However, by focusing on those mechanisms that appear to be of relevance to humans, it may be possible to identify key toxicological responses, which provide a clear indication of carcinogenic potential. In the longer term, the development of more sophisticated, sensitive in vitro detection systems for the endpoints of concern should be of value. The need for a sound database of reference compounds against which to test different approaches and systems cannot be overemphasised.

### 4.2. Developmental toxicity

#### 4.2.1. Introduction

Reproductive toxicology embraces studies on male and female fertility and on developmental toxicity, with special emphasis on embryotoxicity and teratogenicity. Useful reviews have been published (Brown et al., 1995; Spielmann, 1998).

This section focuses on developmental toxicity. Despite increasing knowledge on mammalian development, we still have insufficient insight into normal development to provide a basis for an adequate understanding of the mechanisms of toxic interferences with normal mammalian developmental processes. Nevertheless, over the past 20 years, more than 30 different culture systems have been proposed as tests for developmental toxicity. The majority of these tests have each been used by only one laboratory. The culture systems fall into the following categories:

1. Tests on non-vertebrate species, including *Hydra*, slime moulds, brine shrimps and *Drosophila*.
2. Tests on lower vertebrate embryos or embryonic cell aggregates, including fish, amphibians and birds.
3. Tests on whole mammalian embryos.
4. Tests on micromass cultures from mammalian embryos (limb buds, midbrain).
5. Tests on embryonic stem cells or embryonic stem cell lines.
6. Tests on other mammalian cell lines (e.g. human embryonic palate mesenchymal cells, mouse ovarian tumour cells, neuroblastoma cells, teratocarcinoma cells).

However, where protecting human beings is the aim, it could be argued that these tests are unlikely to gain widespread acceptance and use. The following summary of existing and most promising systems is mainly derived from Spielmann (1998).

#### 4.2.2. Cell lines and embryonic stem cells

A number of established cell lines have been used for screening purposes (Spielmann, 1998). These include: human embryonic palate mesenchymal cells (Pratt et al., 1982), mouse ovarian tumour cells (Braun et al., 1982) and neuroblastoma cells (Mummery et al., 1984). The results of blind trials were not very promising, showing a high number of false positives (Steele et al., 1988).

The use of omnipotent embryonic stem cell lines shows more promising results. For instance, blastocyst totipotent embryonic stem cells (ES) can be cultured under conditions in which the cells form several types of differentiated cells, such as muscle cells or haematopoietic cells (Heuer et al., 1994; Rohwedel et al., 1994). These culture systems can be used to determine the two essential features of embryotoxicity: inhibition of differentiation and/or a higher sensitivity to cytotoxic effects in embryonic cells than adult tissues (Spielmann, 1998). Results of such a test were comparable to the outcome of an embryotoxicity test with rat whole embryo cultures (Spielmann et al., 1997).

The use of ES cells in the production of transgenic cells with targeted mutations and reporter constructs should enable the development of tests with simplified endpoints, which can be used in robotised assay systems.

Although the use of human *embryonic* stem cell lines for developmental toxicity testing might be controversial in some quarters, it is clear that human cells have advantages in predicting effects in humans. Therefore, new developments in which multipotent (or even totipotent) stem cells can be isolated from adult tissues are very promising. For example, nervous tissue stem cells can give raise to haematopoietic stem cells and vice versa (Almeida-Porada et al., 2001).

#### 4.2.3. Aggregate and micromass cultures

Different aggregate and micromass cultures have been used in developmental toxicity tests. One of these systems aggregates of primary cultures makes use of chick embryo neural retina cells (CERC) (Daston et al., 1991). Other systems employ cells from the undifferentiated mesenchyme of early embryo limbs (Umansky, 1966). When these cells were cultured in small volumes at high density, they formed numerous small foci of differentiating chondrocytes within a background of apparently undifferentiated cells. Cell adhesion, movement, communication, division and differentiation all occur in micromass cultures (Umansky, 1966). In principle, the micromass test is based on detecting the ability of a particular chemical to inhibit the formation of foci. Similar systems have been developed with embryonic limb or central nervous system (CNS) cells (usually midbrain, which form foci of neurons) from chick, mouse or rat. The technique has been modified for use with 96-well microtiter plates (Flint, 1993).

#### 4.2.4. Embryos of lower order species

Test systems using embryos of submammalian vertebrate and invertebrate species for detecting the teratogenic potentials of chemicals have been described, including *Hydra*, fish, frogs, crickets, *Drosophila*, brine shrimp and slime mold (Spielmann, 1998). Several of these are currently being used extensively as models for investigating mechanisms of development. Because any stage or component of development is a potential target for toxicants, the existence of species differences is a strong argument in favor of using vertebrate models for predictive screening. However, subvertebrate systems may have applications in ecotoxicologic monitoring. An example is the so-called FETAX test (frog embryo teratogenesis assay xenopus) (Bantie et al., 1990). The assay is limited by aqueous solubility of test substances and the smaller number of laboratories that have the system.

The US Interagency Committee for the Validation of Alternative Methods (ICCVAM) is currently evaluating the validation status of this test.

#### 4.2.5. Avian and mammalian whole embryo culture

Avian embryos are widely used as models in developmental biology. One test system has been developed for use in embryotoxicity testing: the chick embryotoxicity screening test (CHEST) (Jelinek, 1977). Although this system is relatively easy to handle, one general problem with CHEST has been the inability to distinguish general toxicity from specific developmental effects (Jelinek, 1977; Jelinek et al., 1985).

Mammalian embryos can be maintained in culture for short periods throughout the phase from fertilization to the end of organogenesis (Spielmann and Vogel, 1989). Screening systems using mouse (Sadler et al., 1982), rat (Schmid, 1985) and rabbit (Ninomiya et al., 1993) embryos have been described. For toxicity testing, the period from the end of gastrulation to mid-organogenesis has been investigated extensively. At the end of the culture period a number of endpoints can be measured, including effects on the development of the visceral yolk sac vascularization and circulation; effects on haematopoiesis, embryonic growth, differentiation (number of somites, morphologic score); and dysmorphogenic effects (Sadler et al., 1982).

These embryo culture systems are well developed and widely used for the detection of potentially teratogenic compounds and for the elucidation of mechanisms of teratogenicity. They allow the detection of dysmorphogenesis in many organs and the comparison of specific dysmorphogenic effects with general adverse effects on growth and differentiation. In addition, they enable the potencies of structurally related compounds to be ranked. Concentrations of test compounds and metabolites can easily be monitored in the culture medium and embryonic tissues (Spielmann, 1998).

Limitations of these systems are related to the fact that they are relatively complex, cover only a part of organogenesis, require high technical skills and they also can be costly. Whether the use of mammalian tissue and serum is justified for screening purposes may be evaluated by including it in comparative trials with other, simpler in vitro systems (Spielmann, 1998).

*4.2.6. Validation*

A number of validation studies have been carried out (Piersma et al., 1995). In a validation study on different culture systems, six pairs of coded compounds were tested in chick and rat embryo cultures and in brain cell aggregate cultures (Kucera et al., 1993), showing an excellent agreement between in vivo and in vitro data for a series of retinoids (Spielmann and Liebsch, 2001). In the European Union (EU), there is a strong demand for validated in vitro tests in developmental toxicity testing using mammalian embryos as well as primary cultures of embryonic cells and permanent cell lines. During the years 1997–2000, three in vitro embryotoxicity tests were subject to validation using 20 test chemicals which were well characterised by high quality in vivo data in humans and animals. Each in vitro test was evaluated under blind conditions in four laboratories. The most important outcome was that, for the first time, three in vitro embryotoxicity tests have been established that are backed by validated test protocols. They include the whole embryo culture test using cultures of whole rat embryos, the micro mass (MM) test employing primary cultures of dissociated limb bud cells of rat embryos, and the embryonic stem cell test (EST), using two established mouse embryonic cell lines not requiring the sacrifice of pregnant animals. All of the in vitro embryotoxicity tests meet three essential criteria of validated alternative toxicity tests. First, standard operation procedures (SOPs) were established, which are now available to the public. Secondly, sound biostatistical prediction models (PMs) have been established and validated (Genschow et al., 2000). The PMs for all of the three tests provide an overall accuracy close to 80% and, more importantly, 100% predictivity for strongly embryotoxic chemicals. Thus, they can routinely be used to identify strongly embryotoxic chemicals, for instance when screening new substances. Thirdly, the three in vitro tests were experimentally validated in a blind ring trial according to a validation scheme recommended by the EU, the OECD and the US NIEHS (Balls et al., 1990, 1995; OECD, 1996; ICCVAM, 1997b). The full results of this study will be published in 2001.

*4.2.7. Future developments*

The production of a direct effect on the developing organism depends on the concentration/time relationship of the chemical and/or its active metabolite(s) in the target cells (Spielmann, 1998). Therefore, toxicokinetic and metabolism studies are of crucial importance for the design and interpretation of developmental toxicity studies with both in vitro and in vivo methods (Nau, 1990; Andrews et al., 1995). In vivo target concentrations are dependent on maternal absorption of the compound, its distribution, metabolism and excretion, and its placental transfer and distribution in the embryo. For the in vitro systems, the use of physiologically-based toxicokinetic modelling and the integration of the outcome with in vitro measurements of effects may be of great use in the future (Clewell et al., 1997; Blaauboer et al., 2000).

Additionally, a number of developments in the field of in vitro developmental toxicity testing are worth noting.

First, the embryonic stem cell test is being further developed to cover all three main embryonic cell types (endoderm, mesoderm, ectoderm), and to involve more sophisticated endpoints, as well as the use of genetically modified cells.

Secondly, consideration is being given to the ethical use of human embryonic or post-fatal stem cell lines. It is very difficult to extrapolate the outcomes of any developmental toxicity test in animals to humans because of species differences. This applies even more to in vitro tests based on laboratory animal systems because they need additional validation concerning in vitro to in vivo extrapolation. It is to be hoped that this difficulty could be solved, at least in part, by the development of tests based on the ethical use of human stem cell lines. Thirdly, it also needs to be considered how the validated methods could be used for various purposes, for example in candidate compound selection or for regulatory purposes. Fourthly, the final report of an EC-supported study on the development of in vitro mammalian germ cell culture systems and genetic markers for reproductive pharmacotoxicology has been received by DG Research. Although this focuses more on fundamental research it might be of future use for test development. There are limitations to the use of in vitro tests in developmental toxicity in that they have been selected to detect certain structural alterations (Spielmann, 1998). It would be a future need to find tests that may have a wider implication, for example, for the more quantitative parameters such as fetal weight and other functional abnormalities, also taking into account the kinetic considerations. The use of whole embryo cultures or the stem cell approach described above is a promising field of future research. Thus, these limitations of in vitro assays may be manageable by test selection and design. In vitro systems can include specific metabolising systems or be manipulated to approximate the kinetic behaviour of the test agent in the species of interest. In fact, the ease with which such test systems can be manipulated may in some cases mean that limitations can be turned into advantages.

### 4.3. Prediction of allergenicity

#### 4.3.1. Introduction

A large number of compounds are known to adversely modulate the immune system, so as to alter its responsiveness. Compounds having this potential include many low molecular weight chemicals (LMWC) such as pharmaceuticals, occupational chemicals and environmental pollutants, but also larger compounds such as food allergens and biological products (vaccines and blood products isolated from biological sources) and biotechnology-derived pharmaceuticals (e.g. peptides manufactured by recombinant DNA techniques, monoclonal antibodies and gene-therapy products).

Immunological alterations that can be observed vary from immunostimulatory to immunodepressing effects, and even combinations of these. In the case of immunodepression or immunosuppression, cytotoxicity towards components of the immune system is often the cause of a less effective immune response towards an infectious agent.

The main adverse effect that might result from stimulation of the immune system is allergy. Allergy in this respect can be subdivided into the following non-mutually exclusive main categories: contact allergy, respiratory allergy, drug allergy and food allergy. The nomenclature of these categories is somewhat confusing since it is based both on organ-specific and on compound-specific terminology. It may become even more confusing if one realizes that contact as well as respiratory allergy can be induced by occupational chemicals, and that food allergy may result in skin reactions (atopic dermatitis) or respiratory allergic reactions. Drug allergy may result in systemic autoimmune-like symptoms, (sometimes referred to as systemic allergy), and may involve respiratory and skin effects as well.

It is important to note that most compounds (chemicals as well as proteins) induce immunological tolerance when given or taken orally. Conceivably, the incidence in the general population of food allergy is about 2% (Ortolani et al., 2001) or even very low, as in the case of drug allergy. As the incidence of food allergy in children is higher (8–10%), oral tolerance is apparently less well developed in children.

Food allergens are mostly glycoproteins of high molecular weight, further designated as high molecular weight chemicals (HMWC). Allergenic responses to LMWC induced via the oral route are rare but may nevertheless occur, as evidenced by the phenomenon of drug allergy on oral intake. This may be of relevance with respect to undesired or uncontrolled contamination of food by veterinary use of drugs or antibiotics (e.g. penicillin). Only few reports (Wilson and Scott, 1989; Fuglsang et al., 1993) have shown some cases of adverse clinical reactions to food additives such as preservatives and colours. These reactions were characterised as indicative of food intolerance (defined as a non-immune mediated hypersensitivity reaction), although serum antibody levels were not analysed and therefore allergy cannot be formally excluded from these studies.

*4.3.1.1. Basics of T cell sensitisation.* Allergy implies that T lymphocytes are activated and sensitised. For this, the T cell has to recognise the allergenic material in the context of an MHC-II + peptide complex on an antigen-presenting cell (which in a naive individual is a macrophage-like dendritic cell). In addition to antigen recognition, adjuvant signals are required for optimum T cell sensitisation. Inflammatory signals (cytokines such as IL1β, TNFα and IL6) are considered important adjuvant signals, and may result in indispensable extra or so-called co-stimulatory help to T lymphocytes.

The discrimination between LMWC and HMWC is important, since LMWC, in contrast to HMWC, cannot be recognised as such by the immune system. In order to become immunogenic, LMWC need to conjugate to a carrier protein (hapten-carrier mechanism), which can be any protein in the body. HMWCs, in particular peptides derived from these, can be recognised by T cells, in the context of MHC molecules. Some LMWC need to be metabolically converted to a reactive hapten, and these are called prohaptens.

Once a T cell is activated and sensitised, memory immune responses to the same allergenic material may take place. These memory responses are driven by memory T lymphocytes and these need to differentiate from naive T cells under influence of a number of factors (among others cytokines) over a certain period of time. In mice, memory responses are assessed 5–15 days or even several weeks after initial administration of the antigenic compound and this time is required for optimum read out or to obtain clear distinction between effects due to primary and memory response. The main characteristic of a memory response is that it occurs faster and more efficiently than a naive immune response.

Whether and how exactly memory responses as a result of immunosensitisation ensue depends largely on a number of predisposing, often inherently linked, factors, such as MHC haplotype, sex hormones, ongoing infections, health status, etc. In the case of respiratory allergy, but also food allergy and, to some extent, contact allergy (e.g. atopic dermatitis) the genetically determined susceptibility to respond with the IgE-isotype of antibody (also called atopy) is of major relevance.

Overall, immunostimulation leading to one of the different forms of allergy is a very complex process in which many factors are not completely or at best partly understood. This makes prediction of full allergenic potential of substances by means of in vitro tests extremely difficult.

*4.3.2. State of the art*

Although some promising candidate models are under study (Barlow et al., 2002), in vivo models for food allergy are not yet available. Models to assess allergy by LMWC via the oral route are also not available. Development of such models is hampered by the lack of fundamental knowledge on the mechanisms that underlie tolerance induction via the gastrointestinal immune system. Understandably, in vitro models to predict orally induced allergies or even aspects of that are not available. In vitro tests that are available for prediction of allergy originate from contact allergy research, but none of these is as yet validated. As it might be worthwhile to investigate whether these methods are also applicable to assess allergenicity of LMWC in the food, they are summarised below.

*4.3.2.1. Applicability and limitations of in vitro testing.*

- In vitro methods for contact allergy may be applicable for identification of some aspects of foodborne allergenic LMWC and also illustrate some important aspects of immunogenicity. The state of the art of these in vitro models for prediction of sensitising potential of LMWC is briefly reviewed.
- Structure–activity relationship approaches taking into account lipophilicity and reactivity to standard nucleophilic groups (Roberts and Williams, 1982). These methods are able to predict the allergenicity of structurally related chemicals.
- Testing of adjuvant activity: including production of proinflammatory cytokines by freshly isolated cells or cell lines such as fibroblasts or keratinocytes in case of the skin and activation of antigen presenting cells or dendritic cells (expression of activation molecules such as CD54, CD80, CD86, MHC molecules). These methods may be relatively simple but strong reactivity of certain LMWC may interfere by causing cell death.
- Stimulation of T cells: although several attempts (Coates and Walker, 1992; Yokozeki et al., 1995; Rustemeyer et al., 1998, 1999) have been made to stimulate naive T cells in a LMWC-specific manner, methods that have been used appeared very complicated, involving difficult isolation procedures and culture conditions (Hauser and Katz, 1988). In addition, the scarcity of specific T cells in a naive immune system (Borghans et al., 1999) and the often strong reactivity of the LMWC further hamper these test systems. Ex vivo restimulation of (memory) T cells (Stejskal et al., 1986; Griem et al., 1996; Kohler et al., 1997; Padovan et al., 1999) seems a more feasible approach as the number of specific T cells is enhanced considerably on in vivo sensitisation and the response can be elicited with a non-toxic hapten-carrier conjugate, such as LMWC coupled to bovine serum albumin) (Goebel et al., 1995).

Obviously, these ex vivo methods to detect memory T cells are not complete alternative methods, but a positive response demonstrates the actual presence of memory lymphocytes and such tests would exclude the need to challenge or re-treat animals with the same chemical to elicit and detect a memory immune response.

*4.3.2.2. Detection of sensitising potential of HMWC in the food.* General information on food allergy is provided in Barlow et al. (2002). This review concentrates on alternative methods that are available for assessing some aspects of food allergens.

A decision tree strategy to assess the allergenic potential of foods, in particular when derived from genetically engineered plants has been designed (Metcalfe et al., 1996; FAO/WHO, 2001). In this decision tree, the strategy of how to consider the new food product depends on whether the newly engineered food component contains a gene from a source that has documented allergenic potential in man. If it does, the product must be subjected to a number of in vitro and/or in vivo immunological tests for allergenic potential of the new product. These tests include solid phase immunoassays, skin prick tests and double blind placebo-controlled food challenges (DBPCFC). Alternatively, if the gene is derived from a source that is not associated with allergy in man the strategy follows tests to assess sequence similarities, and stability tests to digestibility or processing. If the outcome of these tests is indicative of potential allergenicity animal tests should follow.

In the decision tree, the following items can be considered relevant for alternative testing of genetically engineered products:

- Solid phase immunoassay

Assessment of the presence of allergenic components in food can be done by the radioallergosorbent test (RAST) or RAST inhibition assay, or by ELISA methods. In the proposed decision tree approach these assays use IgE-positive sera from at least 14 individuals that are allergic to the food from which the inserted gene is derived. Any positive detection in these tests requires labelling to the source of the gene.

- Sequence similarity

The part of a protein that is recognised by a B or T cell is called an epitope, and since IgE induction

depends on T cell help, knowledge of the presence of the dominant epitope in a food component is considered of crucial importance. T cell epitopes consist of a primary peptide part of at least eight amino acids in length and are recognised in the context of MHC molecules on APC. This of the amino acid sequence of a new protein with that of known allergens and a match of at least eight contiguous identical amino acids may indicate allergenic potential. For B cell epitopes, which are usually of tertiary structure, this approach is limited. This will be a problem in particular for already sensitised individuals as they possess specific IgE molecules that can recognise these tertiary structures.

- Stability to digestion/processing (Astwood et al., 1996)

A protein that is stable to proteolysis has an increased probability to reach the intestinal mucosa and subsequently the immune system of the intestine. As a result, this protein may be more allergenic than proteins that are completely digested. Digestibility testing can be done using simulated gastric and/or intestinal fluid models. Stability is assessed as the time that the protein remains intact under the digestive conditions. The strong food allergens appear stable for the whole period of 60 min, whereas common food proteins rapidly degrade within 15 seconds.

#### *4.3.3. Future prospects for the premarket hazard identification*

Although the in vitro methods mentioned here may be helpful to assess some isolated aspects of allergenicity of foodborne LMWC and HMWC, one should keep in mind that positive identification by these methods does not necessarily imply that such compounds will indeed be allergenic in vivo. As noted earlier, most foodborne antigenic compounds induce immunological tolerance due to suppressive or regulating properties of the mucosal immune system. Evidently, more fundamental research into aspects of mucosal immunity of the gastrointestinal tract is needed to fill gaps in the knowledge of allergic responses to food components such as new genetically engineered food products. Overall, prediction of hazardous allergenic responses as such currently relies on whole animal studies (Barlow et al., 2002).

## 5. In vitro approaches for development of biomarkers

### *5.1. Introduction*

The following is a summary on the utilisation of biomarker approaches for analysing the complex in vivo impacts of food-associated chemicals or dietary ingredients, mainly to elucidate cancer risks. However, it is equally important to develop biomarkers for non-cancer endpoints (see also section 2.2 on Cellular responses).

A major aim is the identification of potential hazards of food chemicals and their metabolites formed in the body. As the impact of toxic factors greatly depends on dietary antitoxic substances as well, similar attention must be given to biomarkers indicative of protection and of susceptibility. Techniques developed for hazard identification include biomarkers of exposure, such as analytical determinations of chemicals in the body and biomarkers of effect, for example genetic lesions in somatic cells. These biomarkers, measured in body fluids (urine or plasma) and in somatic cells such as lymphocytes, can reveal an overall load of genotoxic burden in the body. More relevant approaches are seen in utilising somatic cells in which the disease arises. Newer developments are aimed at understanding the impact of hazards as influenced by individual susceptibility. Types of susceptibilities currently utilised as biomarkers include those indicating the presence of predetermining damage, that is, alterations of tumour associated genes and those increasing susceptibility to damage, for example polymorphisms of DNA repair enzymes, and enzymes of xenobiotic metabolism. Such susceptibility is, however, not only influenced genetically, but is additionally governed by the impact of exogenous exposure, diet representing the prime parameter that markedly affects susceptibility. Some functional biomarker techniques have been reported that are used more or less successfully to show effects of foods or food products, studying the mucosal immune, which are considered to increase the risks of developing cancer and others that may decrease risks. These include the classical sets of toxicological biomarkers used as tools in food research (Crews and Hanley, 1995; Bottrill, 1998). However, there is much need for further development of methods to increase understanding and to enhance predictivity.

In this context in vitro toxicology assays could further aid in biomarker development by being used for:

- Analysis of mechanisms of effect
- Studies of combination effects (synergism, inhibition)
- Understanding how susceptibilities may influence impact of risk factors
- Finding new endpoints.

Additionally, in vitro methods may be further developed to serve as parts of the biomarker techniques such as:

- Performance of challenge assays with lymphocytes
- Analysis of faecal water genotoxicity in colon cells
- Isolation of exfoliated cells as targets in biomarker determination

- Use of cells from biopsies as targets in biomarker determination.

### 5.2. State of the art and potential role of in vitro tests

#### 5.2.1. Definition and role

Biomarkers are tools used in human studies to assess exposure and disease risks (see this section, cancer). Many definitions of biomarkers have been described and published (Benford et al., 2000). For the purpose of this section, a modified version of Bottrill's definition is employed in which biomarkers are defined as "*parameters which can be evaluated quantitatively, semi-quantitatively or qualitatively and which provide information on exposure to a xenobiotic or on the actual or potential effects of that exposure in an individual or in a group*" (Bottrill, 1998). Thus, the biomarkers discussed here are mainly related to the impact of exogenous factors for cancer risks and are not identical to those dealing with the early detection of the cancer disease (Pepe et al., 2001). For hazard identification of food-associated chemicals, it must be borne in mind that their impact on an organism will depend on the individual state of chemoprotection as well as on specific characteristics of genetic susceptibility. The new research area of "Pharmacogenetics" acknowledges that an individual's response reflects the interaction of their genes with the environment. Specifically, "Pharmacogenetics", is defined as "*the study of how genetic differences influence the variability in individual response to environmental exposure*" (Roses, 2000). Although the field has originally developed from research with drugs, it is well recognised that similar principles should apply to the toxicology of food constituents and in nutrition research.

### 5.3. Types of biomarkers

#### 5.3.1. Biomarkers of exposure

Chemical analytical determination of the compound under study, its metabolites or specific reaction products with macromolecules (e.g. DNA or protein adducts) can be used as exposure biomarkers (Haseltine et al., 1983; Schut and Shiverick, 1992). The objective of such methods is to detect toxic compounds circulating in plasma or excreted in urine, as a reflection of the systemic exposure load (see Dybing et al., 2002; Kroes et al., 2002). Other examples include the detection of DNA-reactive metabolites (Perera and Whyatt, 1994; Arif and Gupta, 1997), or the excretion of endogenously formed compounds in the urine (Wu et al., 1993). Markers of oxidative stress, such as malondialdehyde–DNA adducts (Fang et al., 1997), malondialdehyde plasma levels (Nielsen et al., 1997) and oxidised DNA bases (Nakajima et al., 1996) may also be used as biomarkers for prevention of specific diseases, such as some forms of cardiovascular disease (Machlin, 1995). In addition, exposure can also be determined functionally, that is, by assessing toxic or genotoxic impacts of body fluids, mutagenicity in cell or tissue culture and in body fluids (Hayatsu et al., 1985). It is this latter type of approach that will benefit greatly by receiving input from in vitro toxicology methods.

*5.3.1.1. Challenge assays to assess impacts of body fluids in model cell cultures.* In this case, in vitro toxicological assessment of food-associated compounds with cell culture techniques will reveal or disclose new and sensitive parameters that can be used for studying the impact of body fluids towards cells representing the target tissue of interest. An example is to determine fecal water genotoxicity in cultured human colon cells as a measure of exposure to risk factors in the gut lumen (Venturi et al., 1997; Rieger et al., 1999). Thus, virtually any of the endpoints that are indicative of cellular responses (see section 2.2 on Cellular responses) by a specific hazardous food chemical may reveal whether the body fluid contains the chemical, causing a certain type of cellular response or group of responses. A pattern of selected effects (see sections 2.2 and 3.1 on Cytotoxicity, genotoxicity, cancer-related endpoints) known to be induced by the hazardous chemical under study in vitro, can be analysed after incubation of the same cell types with the body fluid of potentially exposed individuals. This type of approach has already been used to determine how dietary intervention will lead to altered levels of bile acids in faecal water (Venturi et al., 1997), whether dietary antioxidants will decrease the impact of hydrogen peroxide or modulate other cellular parameters (Duthie et al., 1996), or whether luminal contents contain factors that could modulate gene expression in colon epithelial cells (Glinghammer et al., 1999). These studies with body fluids can be performed with all types of cells potentially exposed to body fluids using appropriate cell culture models. For example, colon cells would be used for testing fecal water, bladder cells for urine, lymphocytes for plasma samples. Another possibility is to simulate the exposure situation in vivo, for analysing effects in the gut. Here, longer-term gut fermentation systems are available that can be analysed in vitro in analogy to body fluids. This also allows the question of how hazardous food chemicals can be affected by gut metabolism in cells to be addressed, and how this may alter their impact on cells of the gastrointestinal tract. Hence, it is to be anticipated that it will be possible to identify parameters useful in faecal biomarkers assays.

#### 5.3.2. Biomarkers of effect

Specific DNA adducts, DNA breaks, oxidative DNA damage, micronuclei (e.g. in blood lymphocytes and selected somatic cells) may be indicative of an increased

hazard. This is based on the assumption that increased DNA damage will enhance the probability of mutations occurring in critical target genes and cells, and/or that increased DNA damage is the result of a higher load of genotoxic agents which will enhance the process of carcinogenesis (by inducing DNA damage as well as other molecular processes of carcinogenesis). The damage may be detected in peripheral blood lymphocytes, in any primary or cultured cell system, or by analysing the excretion in urine or plasma of reaction products indicative for genotoxic interactions. In a few cases the detection is also possible in tumour target tissues or in tissue associated specific fluids (e.g. colon biopsies or faeces; exfoliated bladder cells or urine).

Numerous assay methods are available, such as microsatellite alterations (Mao et al., 1994), mutagen hypersensitivity (Olden, 1994), micronuclei in lymphocytes (Fenech and Rinaldi, 1995), chromosomal aberrations, proliferative rate index (Anderson et al., 1988) and human somatic mutations (Compton et al., 1991). Other techniques can be used to study biomarkers of effect, but as noted below they can also be used to analyse exposure. These include $^{32}P$ postlabeling (Beach and Gupta, 1992) methods to detect specific DNA adducts (Yadollahi-Farsani et al., 1996), and various methods to determine oxidative damage in peripheral blood lymphocytes, plasma, urine or sputum.

Non-invasive biomarkers, which may be used to assess the overall body load of genotoxicants, are based on determining genetic alterations. The development of the techniques for genetic damage has, however, been largely driven by their utilisation as methods to assess exposure in occupational or environmental settings (Haseltine et al., 1983; Albertini and Hayes, 1997; Arif and Gupta, 1997; Tucker et al., 1997; Wild and Pisani, 1997; Au et al., 1998).

The methods may utilise lymphocytes as target cells. However, many of these markers are expected to give more relevant information if they are determined in cells from biopsies obtained from the actual target tissues. As the biopsy approach is invasive, the application may be limited to special exposures or disease cases. In any case, their utilisation and development will serve as the basis for refinement of non-invasive methods using exfoliated cells, from urinary tract, oral mucosa, faeces, breast epithelium, etc.

*5.3.2.1. Human tissues as targets for biomarkers and to identify new parameters of effect*. Many of these parameters correspond to responses evoked by hazardous chemicals in cell culture experiments. Thus, the in vitro toxicological assessment of food-associated compounds in cell culture is hoped to reveal or disclose new and sensitive parameters that can be employed as biomarkers to analyse the impact in vivo after ingestion of food containing the chemical. Basic expertise from in vitro toxicological assays will also be needed for work using biopsies or exfoliated cells. Here the "know how" and specific technical competence that are a result of working with different cellular systems is to be applied as a prerequisite for using human cell material to analyse parameters, which may indicate specific effects. An example of a biomarker that requires expertise in cell or tissue culture is the determination of cell proliferation in rectal or colonic crypts (Bartram et al., 1993). In this context, it can also be envisaged that additional, newly developed endpoints of cytotoxicity, cancer-related endpoints, cellular responses (see sections 2.1 and 2.2) will offer new avenues for the development of new biomarker assays as for example, the inducibility of hemoxygenase (Menzel et al., 1998), heat shock factors (Hosokawa et al., 1992) or inhibition of DNA repair (Duthie and Collins, 1997). One of the most promising approaches, however, will be to compare profiles of complex gene or protein expression (see section 2.2.1 on Genomics and proteomics) in cells in vitro. Differences of the respective profiles of exposed and non-exposed cells can be used to develop biomarkers. For example, exposure to nickel salts leads to different patterns of gene activation (Zhou et al., 1998) or gene silencing (Klein and Costa, 1997) as compared to control cells, and the identification of such different profiles could be utilised to identify endpoints in biomarker approaches to identify in vivo exposure in humans.

*5.3.3. Susceptibility biomarkers*

In the future it will be more and more important to analyse the impact of food hazards on the basis of genetic and other susceptibilities. Three main types of susceptibilities for cancer can be distinguished:

- Predetermining damage: One possibility is to identify individuals at high risk, since they carry high penetrance genetic alterations in cancer target genes (see van den Brandt et al., 2002). The further development of biomarkers could include the isolation of cells from these individuals to search for additional associated parameters, which could enhance the detection of alterations at very earlier stages (e.g. specific adducts in p53 genes).
- Predisposing alterations: The second possibility is to identify individuals at different degrees of risk because they carry frequent low penetrating alterations in genes (genetic polymorphisms), which occur frequently and are more indirectly related to the process of carcinogenesis (see van den Brandt et al., 2002). These include the genetic polymorphisms for activating enzymes (which usually catalyse oxidation reactions, phase I enzymes). These genetic variants may be associated with either enhanced or decreased

rates of metabolic conversion by the specific enzyme. Depending on the type of metabolic conversion (activation, deactivation), the result will be more or less genotoxic exposure and thus risk.

- Diet and metabolic balance: Future biomarker approaches will consider more and more modulating influences of the diet as a susceptibility factor and thus on biomarker responses. Some influences may lead to increased genetic damage, making the cells more vulnerable to additional toxic compounds. Some may lead to less damage, making the cells more tolerant to additional exposure related factors. Reported examples are higher levels of 5-HO-methyl uracil by high fat diets (Djuric et al., 1991) or modulation of malondialdehyde–DNA adducts by diets with different fatty acid composition (Fang et al., 1997), or reduction of intrinsic oxidative DNA damage by moderate wine consumption (Fenech et al., 1997). Recently, the adequate consumption of carotenoid containing vegetable juices (Pool-Zobel et al., 1997) or intake of vitamins as dairy supplements (Duthie et al., 1996) have been shown to reduce oxidative damage of lymphocyte DNA. In contrast, the comparison of vegetarian and non-vegetarian lifestyles did not show differences in genetic damage in lymphocytes, detected as micronuclei (Fenech and Rinaldi, 1995; Kim and Mason, 1996; Perera, 1996). Modulation of metabolising enzymes by dietary influences are expected to affect the impact of hazardous food compounds.. Thus, the dietary induction of glutathione *S*-transferases may be considered a protective mechanism in situations of exposure to hazardous chemicals which are deactivated by these enzymes.

*5.3.3.1. Studying the impact of hazardous chemicals on the basis of susceptibility factors*. In vitro tests may be used to enhance our understanding of how specific predetermining genetic alterations or patterns of genetic enzyme polymorphisms can affect the impact of a particular hazardous compound under study (see section 3.1 on Cancer-related endpoints). This can be easily achieved by determining the toxicity of a given compound in cells containing the specific genetic alteration (e.g. repair deficiency) or patterns of enzyme polymorphisms such as wild-type vs deletion polymorphisms for certain glutathione *S*-transferases. More refined methods using genetically engineered cells highly expressing or lacking a specific gene are already used in these types of studies to gain more information on the consequences of susceptibilities for hazard evaluation.

A forward-looking approach would be to utilize endpoints identified using the techniques of genomics/transcriptomics and proteomics (see section 2.2 on Cellular responses). It is possible to induce complex patterns of gene expression with individual dietary components and to compare the susceptibilities of treated cells with those of non-treated cells by analysing different profiles of gene expression. The impact of the chemical as a hazard can be measured first by determining basic cytotoxicity (see section 2.1 on Cytotoxicity), or standard parameters of genotoxicity (see section 3.1 on Cancer-related endpoints) in cells with different profiles of gene expression. Preferably, these studies should be performed with the cells that are also usually and easily targeted in biomarker analysis, such as peripheral lymphocytes, since this will facilitate the extrapolation of the in vitro data to the in vivo situation. An even more sophisticated approach could involve the determination of various parameters of cellular response in cells of tumour target tissue to understand the consequences of hazardous chemicals on the basis of complex susceptibility profiles.

### *5.4. Conclusion*

Biomarkers for detecting toxicity, in particular cancer-related endpoints, after ingestion of hazardous food compounds are available. Many of these, however, need to be validated for their applicability, reliability and predictivity. Moreover, biomarker techniques useful for identification of potentially causative hazards for diseases other than cancer are much less advanced. Cancer-related endpoints connected to oxidative stress, however, might be relevant also to hazards leading to other degenerative diseases, including cardiovascular afflictions or other types of diseases potentially connected to oxidative stress.

In vitro toxicology is highly promising for further developments in this field, now that a set of biomarker techniques is available for the assessment of parameters indicative for mechanisms of action. The utilisation of new innovative technologies, such as genomics, transcriptomics and proteomics will allow identification of in vitro clusters of genes and proteins that can be induced or silenced by hazardous food chemicals and this will significantly improve the understanding of underlying mechanisms. Moreover, the identification of specific patterns of response will allow us to measure and characterise results from in vivo exposure in an unprecedented manner. Of equal importance is to additionally evaluate the impact of hazardous chemicals on the basis of intrinsic susceptibilities. This type of integrated, mechanism-driven in vitro to in vivo approach will extensively rely on the increasing use of cell assays to develop new biomarkers in order to enhance our understanding of effects that will arise in vivo.

## 6. General summary and conclusions

In vitro systems such as subcellular fractions, intact cells, tissue slices and organ cultures should play a central role in a general scheme for evaluating the hazards posed by food constituents. In common with other methods used for risk assessment purposes they have, however, their characteristic weaknesses and strengths.

### 6.1. Weaknesses

Cell lines in culture, which are commonly used in many in vitro studies, are usually in vitro derived from tumours and are thus transformed. Human cells derived from normal tissues need to be immortalized. In other in vitro approaches primary cells are used. These cells can only be kept in culture for a certain time (hours to days) and in many cases will lose their differentiated properties during this culture time. These intrinsic weaknesses result from the fact that, in vivo, the cells are isolated from their natural environment and are no longer integrated into an ordered tissue and organ topology. This entails intrinsic limitations concerning reduced survival, imbalance of metabolic competence, and lack of cell–cell interaction, destroyed organ topology and absence of tissue communication. Metabolic conversions of xenobiotics will be studied using subcellular fractions, as a first approach. These systems usually favour only the specific biotransformation step, depending on the type of isolation procedure, on the cofactors added, on the source of tissue, and on the expression level of the involved enzymes. However, the balance of metabolic activation and inactivation requires a highly ordered interplay of many enzymes and cofactors in most cases.

One possible procedure to overcome some of these limitations would be to use recombinant human enzymes or expression systems to provide suitable metabolic systems where necessary and to define enzyme specificity, or to use more integrated systems, such as cells or tissues. One of the major difficulties is in determining the target cell concentration, that is, the absence of effective toxicokinetic data. However, in vitro approaches are being developed that may help overcome this problem. Also, it is difficult to study effects on integrated and at the same time diffusely organised systems like the neuro, immuno and endocrino systems. Hence, to study effects on these organ systems in vitro is not an easy task.

### 6.2. Strengths

Despite these limitations, in vitro systems are extremely useful in many ways. They can provide much more refined information on how food constituents interact with human cells and macromolecules. In vitro test systems are especially well suited for studying low molecular weight chemicals such as food additives, flavourings, substances used in the production of foods, contaminants, pesticide and veterinary residues, and natural toxicants. Additionally, micronutrients and nutritional supplements such as vitamins, minerals, and non-nutritive, bioactive compounds lend themselves to in vitro assessment, especially when mechanistic questions are to be addressed. It also is possible to critically assess effects of complex mixtures to elucidate combination effects in a focused way. In contrast, the determination of properties of macronutrients, such as proteins, carbohydrates or fats, is usually compromised by their bioavailability to the cells. For those cases, special in vitro co-incubation procedures like two-compartment systems provide new avenues of method development. For the investigation of whole foods, it is usually necessary to prepare extracts or concentrates. This also applies to studies on food processing-mediated effects, including traditional processes such as cooking, smoking or drying, as well as technologies such as irradiation or novel processes such as high-pressure treatment. For studying dietary fibre-like ingredients including novel prebiotic food constituents, it may additionally also be feasible to prepare fermentation extracts simulating bacterial metabolism, for example by the gut microflora. Increasingly, it is not sufficient to consider the effects of potentially hazardous food components on their own. Adequate consideration of food matrix components is also necessary. The consideration of chemical structure might also suggest the requirement of modelling specific types of in vivo metabolism such as effected by gut enzymes and gut flora or other potential extra-hepatic metabolism. By focusing on those mechanisms that appear to be of relevance to humans, it should be possible to identify key toxicological responses with high predictive value. Another aim is to study interactions between risk factors and protective factors from food. For the new area of evaluating functional foods in vitro methods are of great promise. The future paradigm should be to use appropriate, validated test systems relevant for human health.

### 6.3. Key features of in vitro systems

In vitro systems:

- provide rapid and effective means of screening and ranking chemicals in and from food for a number of toxicological endpoints;
- allow development of mechanism-driven evaluations in human systems;
- provide important tools to enhance our understanding of the hazardous effects of chemicals at both the cellular and molecular level;

- are essential for bridging between experimental animals and humans, and for detailed understanding of the bases of species differences;
- provide well defined systems for studying structure–activity relationships.

They allow targeted investigations on issues that cannot be adequately addressed by other methods such as:

(i) in-depth analysis of mechanisms of toxicity at both the molecular and cellular level, and of both causal and adaptive responses;
(ii) identification of key molecular events that are involved in toxicity, enabling the development of effective biomarkers of effect;
(iii) detailed analysis of the toxicological consequences of genetic variation within the population;
(iv) assessment of cell-specific (e.g. liver, cardiac, kidney, neural, immune system) and, where possible, tissue-specific (e.g. embryo) effects;
(v) establishing the nature of concentration–effect relationships and the existence of effect-specific thresholds in cells from different species and different tissues.

### 6.4. Priority research needs

It is anticipated that in the future more and more emphasis will be placed on in vitro assays to study toxicity. For this it will be necessary to develop, improve, evaluate and validate systems specifically for studying effects of food associated chemicals. This applies especially to:

1. New endpoints of toxicity:
   (a) Identification of molecular markers based on detecting effects at levels of exposure lower than those, which cause pathological response.
   (b) Identification of markers of different stages of the neoplastic process in the target cells of somatic tissues in which (food-associated) tumours arise, including genotoxic effects/mutations in relevant tumour associated genes or in DNA repair genes as well as intermediate endpoints (e.g. proliferation, apoptosis, differentiation).
   (c) Establishment of specific toxicity profiles in order to predict acute, subacute and chronic toxicity, including the effects of repeated, low-dose exposure.
   (d) Establishment of (batteries of) in vitro systems for endpoint that are relevant for target organ or tissue-specific effects (e.g. by measuring changes in functional parameters of cells and tissues or by using transcriptomics/proteomics).
   (e) Use of such information to develop reliable biomarkers of effect or toxicological endpoints for use in the toxicological evaluation of food chemicals.
   (f) Specific target effects should also include effects on developmental processes.
   (g) Development and evaluation of methods to study allergenicity of food components.

   (i) potential effects by low weight molecular compounds, for example on the mucosal immune system of the gastrointestinal tract;
   (ii) new screening essays for high molecular weight compounds and elucidation of mechanism, such as interaction with mucosal immune system;
   (iii) development of ex vivo tests to study (peptide specific) memory T cell responses.

2. Toxicokinetics:
   (a) In vitro systems for evaluating the biotransformation of food compounds.
   (b) Reliable models of barrier functions (e.g. gastrointestinal tract, blood/brain, placenta), including transporter-functions involved in absorption and excretion of compounds.
   (c) Models to study food matrix effects on the absorption of chemicals.

3. Human-based systems of increasing hierarchy, such as:
   (a) Subcellular systems;
   (b) Cellular systems:primary cells from somatic tissues such as non-transformed cells and cells from preneoplastic foci, tumour cells, embryonic cells, genetically modified cells;
   (c) Whole tissues (e.g. surgical samples).

4. Structured approaches for prediction:
   (a) Integrated strategies combining

   (i) prior knowledge on structural alerts;
   (ii) in vitro assays addressing toxicokinetics.

   (b) Parallelogram approach based on comparing in vitro assays with animal and human cells, supported by

   (i) human exposure data;
   (ii) biomarker-based analysis in animals and humans.

5. Reference libraries and in-silico systems containing information on:
   (a) Structure, structural alerts for toxic effects, chemical functionalitites and biotransformation.

(b) Prior knowledge on toxicity data, for example on the profile of toxic activities with relevance for food.
(c) Known susceptibility factors and their influence on the impact of risks.

## References


Aardema, M.J., Albertini, S., Arni, P., Henderson, L.M., Kirsch-Volders, M., Mackay, J.M., Sarrif, A.M., Stringer, D.A., Taalman, R.D., 1998. Aneuploidy: a report of an ECETOC task force. Mutation Research 410, 3–79.

Acosta, D., Anufuro, D.C., Smith, M.T., 1980. Cytotoxicity of acetaminophen and papaverine in primary cultures of rat hepatocytes. Toxicology and Applied Pharmacology 53, 306–313.

Afshari, C.A., Nuwaysir, E.F., Barrett, J.C., 1999. Application of complementary DNA microarray technology to carcinogen identification, toxicology, and drug safety evaluation. Cancer Research 59, 4759–4760.

Albertini, R.J., Hayes, R.B., 1997. Somatic cell mutations in cancer epidemiology. In: Tonniolo, P., Boffetta, P., Shuker, D.E.G., Rothman, N., Hulka, B., Pearce, N. (Eds.), Application of Biomarkers in Cancer Epidemiology. IARC Publications no. 142. International Agency for Research on Cancer, Lyon , pp. 159–184.

Almeida-Porada, G., Porada, C., Zanjani, E., 2001. Adult stem cell plasticity and methods of detection. Review of Clinical Experimental and Hematology 5, 26–41.

Ames, B.N., Gold, L.S., 1992. Animal cancer tests and cancer prevention. Journal of the National Cancer Institute Monographs 12, 125–132.

Ames, B.N., Gold, L.S., 1997. The causes and prevention of cancer: gaining perspective. Environmental Health Perspectives 105, 865–873.

Andersen, M.E., 1991. Physiologically based modelling of organic compounds. Annals of Occupational Hygiene 35, 309–321.

Anderson, D., Jenkinson, P.C., Dewdney, R.S., Francis, A.J., Godbert, P., Butterworth, K.R., 1988. Chromosome aberrations, mitogen-induced blastogenesis and proliferative rate index in peripheral lymphocytes from 106 control individuals of the U.K. population. Mutation Research 204, 407–420.

Anderson, L.M., Jones, A.B., Kovatch, R.M., 1990. Effect of pretreatment with beta-naphthoflavone on tumorigenesis by N-nitrosoethylurea in five mouse strains. Cancer Letters 52, 91–94.

Anderson, N.L., Seilhamer, J., 1997. A comparison of selected mRNA and protein abundances in human liver. Electophoresis 18, 533–537.

Andersson, T.B., Sjöberg, H., Hoffmann, K.J., Boobis, A.R., Watts, P., Edwards, R.J., Lake, B.G., Price, R.J., Renwick, A.B., Gómez-Lechón, M.J., Castell, J.V., Ingelman-Sundberg, M., Hildestrand, M., Goldfarb, P.S., Lewis, D.F., Corcos, L., Guillouzo, A., Taavitsainen, P., Pelkonen, O., 2001. An assessment of human liver-derived in vitro systems to predict the in vivo metabolism and clearance of almokalant. Drug Metabolism and Disposition 29, 712–720.

Andrews, J.E., Ebron-McCoy, M., Bojic, U., Nau, H., Kaviok, R.J., 1995. Validation of an in vitro teratology system using chiral substances: stereoselective teratogenicity of 4-yn-valproic acid in cultured mouse embryos. Toxicology and Applied Pharmacology 132, 310–316.

Aoki, Y., Lipsky, M.M., Fowler, B.A., 1990. Alteration in protein synthesis in primary cultures of rat kidney proximal tubule epithelial cells by exposure to gallium, indium and arsenite. Toxicology and Applied Pharmacology 106, 462–468.

Arif, J.M., Gupta, R.C., 1997. Detection of DNA-reactive metabolites in serum and their tissue distribution in mice exposed to multiple doses of carcinogen mixtures: role in human biomonitoring. Carcinogenesis 17, 2213–2219.

Arturrson, P., Borchardt, R., 1997. Intestinal drug absorption and metabolism in cell cultures: caco-2 and beyond. Pharmaceutical Research 14, 1655–1660.

Artursson, P., Karlsson, J., 1991. Correlation between oral drug absorption in humans an apparent drug permeability coefficeints in human intestinal epithelial (CACO-2) cells. Biochemical and Biophysical Research Communications 175, 880–885.

Ashby, J., 1986. The prospects for a simplified and internationally harmonized approach to the detection of possible human carcinogens and mutagens. Mutagenesis 1, 3–16.

Ashby, J., 1991. Determination of the genotoxic status of a chemical. Mutation Research 248, 221–231.

Ashby, J., Tennant, R.W., 1991. Definitive relationships among chemical structure, carcinogenicity and mutagenicity for 301 chemicals tested by the U.S.NTP. Mutation Research 257, 229–306.

Astwood, J.D., Leach, J.N., Fuchs, R.L., 1996. Stability of food allergens to digestion in vitro. Nature Biotechnology 14, 1269–1273.

Au, W.W., McConnell, H.M., Wilkinson, G.S., Ramanujam, V.M.S., Alcock, N., 1998. Population monitoring: experience with residents exposed to uranium mining/milling waste. Mutation Research 405, 237–245.

Austel, V., Kutter, E., 1983. Absorption, distribution and metabolism of drugs. In: Topliss, J.G. (Ed.), Quantitative Structure Activity Relationships of Drugs. Academic Press, New York.

Balls, M., Blaauboer, B.J., Brusik, D., Frazier, J., Lamp, D., Pemberton, M., Reinhardt, C., Robertfroid, M., Rosenkranz, H., Schmid, B., Spielmann, H., Stammati, A.L., Walum, E., 1990. Report and recommendations of the CAAT/ERGATT workshop on the validation of toxicity test procedures. ATLA 18, 313–337.

Balls, M., Blaauboer, B.J., Fentem, J., Bruner, L., Combes, R.D., Ekwal, B., Fielder, R.,J., Guillouzo, A., Lewis, R.W., Lovell, D.P., Reinhardt, C.A., Repetto, G., Sladowski, D., Spielmann, H., Zucco, F.,, 1995. Practical aspects of the validation of toxicity test procedures. The report and recommendations of ECVAM Workshop 5. ATLA 23, 129–147.

Balls, M., Bridges, J.W., 1984. The FRAME research program on in vitro toxicology. In: Goldberg, A.M. (Ed.), Acute Toxicology Testing: Alternatives Approaches. Mary Ann Liebert, New York, pp. 61–80.

Bantie, J.A., Fort, D.J., Rayburn, J.R., De Young, D.J., Bush, S.J., 1990. Further validation of FETAX: evaluation of the developmental toxicity of five known mammalian teratogens and non-teratogens. Drug and Chemical Toxicology 13, 267–282.

Barlow, S.M., Grieg, J.B., Bridges, J.W., Carere, A., Carpy, A.J.M., Galli, C.L., Kleiner, J. Knudsen, I., Koëter, H.B.W.M., Levy, L.S., Madsen, C., Mayer, S., Narbonne, J.-F., Pfannkuch, F., Prodanchuk, M.G., Smith, M.R., Steinberg, P., 2002. Hazard identification by methods of animal-based toxicology. Food and Chemical Toxicology 40, 145–191.

Barratt, M.D., 1996. Quantitative structure–activity relationships (QSARs) for skin corrosivity of organic acids, bases and phenols: principal components and neural network analysis of extended datasets. Toxicology in Vitro 10, 85–94.

Barratt, M.D., 1997. QSARs for the eye irritation potential of neutral organic chemicals. Toxicology in Vitro 11, 1–8.

Barratt, M.D., Dixit, M.B., Jones, P.A., 1996. The use of *in vitro* cytotoxicity measurements for the prediction of the skin corrosivity potential of acids: a QSAR method. Toxicology in Vitro 10, 283–290.

Barrett, J.C., Ts'o, P.O., 1978. Evidence for the progressive nature of neoplastic transformation *in vitro*. Proceedings of the National Academy of Sciences of the U.S.A 75, 3761–3765.

Bartram, H.P., Scheppach, W., Schmid, H., Hofmann, A., Dusel, G., Richter, F., Richter, A., Kasper, H., 1993. Proliferation of human colonic mucosa as an intermediate biomarker of carcinogenesis: Effects of butyrate, desoxycholate, calcium, ammonia, and pH. Cancer Research 53, 3283–3288.

Basketter, D.A., Dooms-Goossens, A., Karlberg, A.T., Lepoittevin, J.P., 1995. The chemistry of contact allergy: why is a molecule allergenic? Contact Dermatitis 32, 65–73.

Bäärnhielm, C., Dahlbäck, H., Skanberg, I., 1986. In vivo pharmacokinetics of felodipine predicted from in vitro studies in rat, dog and man. Acta Pharmacologie et Toxicologie 59, 113–122.

Beach, A.C., Gupta, R.C., 1992. Human biomonitoring and the $^{32}$P-postlabeling assay. Carcinogenesis 13, 1053–1074.

Benford, D.J., Hanley, A.B., Bottrill, K., Oehlschlager, S., Balls, M., Branca, F., Castegnaro, J.J., Descotes, J., Hemminiki, K., Lindsay, D., Schilter, B., 2000. Biomakers as predictive tools in toxicity testing. The report and recommendations of ECVAM Workshop 40. ATLA 119–131.

Benigni, R., 1992. Relationship between in vitro mutagenicity assays. Mutagenesis 7, 335–341.

Benigni, R., Andreoli, C., Giuliani, A., 1994. QSAR models for both mutagenic potency and activity: application to nitroarenes and aromatic amines. Environmental and Molecular Mutagenesis 24, 208–219.

Bentley, P., Calder, I., Elcombe, C., Grasso, P., Stringer, D., Wiegand, H.J., 1993. Hepatic peroxisome proliferation in rodents and its significance for humans. Chemical Toxicology 31, 857–907.

Blaauboer, B.J., Barratt, M.D., Houston, J.B., 1999. The integrated use of alternative methods in toxicological risk evaluation. ECVAM Integrated Testing Strategies Task Force Report 1. ATLA 27, 229–237.

Blaauboer, B.J., Forsby, A., Houston, J.B., Beckman, M., Combes, R.D., DeJongh, J., 2000. An integrated approach to the prediction of the systemic toxicity using biokinetic models and biological in vitro test methods. In: Balls, M., van Zeller, A.M., Halder, M.E. (Eds.), Progress in the Reduction Refinement and Replacement of Animal Experimentation. Elsevier, Amsterdam , pp. 525–536.

Blaauboer, B.J., Wortelboer, H.M., Mennes, W.C., 1990. The use of liver cell cultures derived from different mammalian species in in vitro toxicological studies: implementation in extrapolation models? ATLA 18, 251–258.

Blake, M.J., Gershon, D., Fargnoli, J., Holbrook, N.J., 1990. Discordant expression of heat shock protein mRNA's in tussues of heat-stressed rats. Journal of Biological Chemistry 265, 15275–15279.

Boorstein, W.R., Ziegelhoffer, T., Craig, E.A., 1994. Molecular evolution of the HSP70 multigene family. Journal of Molecular Evolution 38, 1–17.

Borenfreund, E., Babich, H., Martin-Alguachi, N., 1988. Comparisons of two *in vitro* cytotoxicity assays—the neutral red (NR) and tetrazolium MTT tests. Toxicology in Vitro 2, 1–6.

Borenfreund, E., Borrero, 1984. In vitro cytotoxicity assays: potential alternatives to the Draize ocular irritancy test. Cell Biology and Toxicology 1, 55–60.

Borenfreund, E., Puerner, J.A., 1985. Toxicity determined in vitro by morphological alterations and neutral red absorption. Toxicology Letters 24, 119–124.

Borghans, J.A.M., Noest, A.J., De Boer, R.J., 1999. How specific should immunological memory be? Journal of Immunology 163, 569–575.

Bottrill, K., 1998. The use of biomarkers as alternatives to current animal tests on food chemicals. ATLA 26, 421–480.

Bowtell, D.D., 1999. Options available from start to finish for optaining expression data by microarray. Nature Genetics 21 (1 Suppl.), 51–55.

Braun, A.G., Nichinson, B.B., Horowicz, P.B., 1982. Inhibition of tumor cell attachment to concanavalin A-coated surfaces as an assay for teratogenic agents: approaches to validation. Teratatogenesis, Carcinogenesis, Mutagenesis 2, 343–354.

Broadhead, C.L., Combes, R.D., 2001. The current status for food additives toxicity testing and the potential for application of the Three Rs. ATLA 29, 471–485.

Brown, N.A., Spielmann, H., Bechter, R., Flint, O.P., Freeman, S.G., Jelinek, R.J., Koch, E., Nau, H., Newall, D.R., Palmer, A.K., Renault, J.Y., Repetto, M.F., Vogel, R., Wiger, R., 1995. Screening chemicals for reproductive toxicity: the current alternatives. The report and recommendations of an ECVAM/ECTS workshop (ECVAM Workshop 12). ATLA 23, 868–882.

Brown, P.O., Botstein, D., 1999. Exploring the new world of the genome with DNA microarrays. Nature Genetics 21 (1 Supp), 33–37.

Brown, R., Foran, J., Olin, S., Robinson, D., 1997. Physiological parameter values for PBPK models. Toxicology and Industrial Health 13, 407–484.

Brown, R.P., Foran, J., Olin, S., Robinson, D., 1994. Physiological Parameter Values for PBPK Models. International Life Science Institute (ILSI) Risk Science Institute (RSI), Washington, DC.

Brusick, D.J., 1986. Genotoxicity of hexachlorbenzene and other chlorinated benzenes. In: Norris, C.R., Cabral, J.R.P. (Eds.), Hexachlorobenzene: Proceedings of an International Symposium. IARC Scientific Publications no. 77. International Agency for Research on Cancer, Lyon, pp. 393–397.

Buchner, Journal 1996. Supervising the fold: functional principles of molecular chaperones. FASEB Journal 10, 10–19.

Butterworth, B.E., Bogdanffy, M.S., 1999. A comprehensive approach to the integration of toxicity and cancer risk assessment. Regulatory Toxicology and Pharmacology 29, 23–36.

Butterworth, B.E., Popp, J.A., Conolly, R.B., Goldsworthy, T.L., 1992. Chemically induced cell proliferation in carcinogenesis. In: Vainio, P.N., Magee, P.N., McGregor, D.B., McMichael, A.J. (Eds.), Mechanisms of Carcinogenesis in Risk Identification, IARC Scientific Publications no. 116. International Agency for Research on Cancer, Lyon , pp. 279–305.

Carere, A., Mohn, G.R., Parry, J.M., Sors, A.I., Nolan, C.V., 1995. Methods and testing strategies for evaluating the genotoxic properties of chemicals. EUR15945EN..

Castro, J.A., Diaz Gomez, M.I., Castro, G.D., 1993. Non-genotoxic carcinogens. Approaches to their risk assessment. Biomedical and Environmental Science 6, 71–80.

CEC, 1989. Guidelines on the Preclinical Safety Testing of Medicinal Products Derived from Biotechnology — Commission of the European Communities notes to the applicants for premarketing authorizations. TIBTECH 7.

Cho, H.Y., Hotchkiss, J.A., Harkema, J.R., 1999. Inflammatory and epithelial response during the development of ozone-induced mucous cell metaplasia in the nasal epithelium of rats. Toxicological Sciences 51, 135–145.

Clark, C.R., Walter, M.K., Ferguson, P.W., Katchen, M., 1988. Comperative dermal carcinogenesis of shale and petrolium-derived distillates. Toxicology and Industrial Health 4, 11–22.

Clark, D.E., 1999. Rapid calculation of polar molecular surface area and its application to the prediction of transport phenomena. Journal of Pharmaceutical Sciences 88, 807–814.

Clayson,D.B., Iverson,F., 1996. Cancer risk assessment at the crossroads: the need to turn to a biological approach. Regulatory Toxicology and Pharmacology 45-59.

Clemedson, C., Barile, F.A., Chesne, C., Cottin, M., Curren, R., Eckwall, B., Ferro, M., Gomez-Lechon, M.J., Imai, K., Janus, J., Kemp, R.B., Kerszman, G., Kjellstrand, P., Lavrijsen, K., Logemann, P., McFarlane-Abdulla, E., Roguet, R., Segner, H., Thuvander, A., Walum, E., Ekwall, B., 2000. MEIC evaluation of acute systemic toxicity. Part VII. Prediction of human toxicity by results from testing of the first 30 reference chemicals with 27 further in vitro assays. ATLA 28, 159–200.

Clewell, H.J., Andersen, M.E., 1986. A multiple dose-route physiological model for volatile chemicals using ACSL/PC. In: Cellier, F.D. (Ed.), Languages for Continuous Simulation. Society for Computer Simulation, San Diego, CA.

Clewell, H.J., Andersen, M.E., Wills, R.J., Latriano, L., 1997. A physiologically based pharmacokinetic model for retinoic acid and its metabolites. Journal of the American Acadamy of Dermatology 36, S77–S85.

Coates, M.E., Walker, R., 1992. Interrelationships between the gastrointestinal microflora and non-nutrient components of the diet. Nutrition Research Reviews 5, 85–96.

Cochrane, B.J., Irby, R.B., Snell, T.W., 1991. Effects of copper and tributyltin on stress protein abundance in the rotifer Brachionus plicatilis. Comparative Biochemistry and Physiology 98C, 385–390.

Coggon, D., Barker, D.J., Cole, R.B., Nelson, M., 1989. J Natl Cancer Inst. 81, 1178–1182.

Combes, R., Balls, M., Curren, R., Fishback, M., Fusenig, N., Kirkland, D., Lasne, C., Landolph, J., Le Boeuf, R., Marquardt, H., McCormick, J., Müller, L., Rivedal, E., Sabbioni, E., Tanaka, N., Vasseur Yamasaki, H., 1999. Cell transformation assays as predictors of human carcinogenicity. The report and recommendations of ECVAM Workshop 39. ATLA 27, 745–767.

Committee on Mutagenicity of Chemicals in Food, C.P.a.t.E.C.U., 2000. Guidelines on a strategy for testing of chemicals for mutagenicity.

Compton, P.J.E., Hooper, K., Smith, M.T., 1991. Human somatic mutation assays as biomarkers of carcinogenesis. Environmental Health Perspectives 94, 135–141.

Crespi, C.L., Miller, V.P., 1999. The use of heterologously expressed drug metabolizing enzymes—state of the art and prospects for the future. Pharmacology and Therapeutics 84, 121–131.

Crews, H.M., Hanley, A.B., 1995. Biomarkers in Food Chemical Risk Assessment. The Royal Society of Chemistry, Cambridge.

Cronin, M.T., Basketter, D.A., 1994. Multivariate analysis of a skin sensitization database. SAR and QSAR in Environmental Research 2, 159–179.

Dai, R., Pincus, M.R., Friedman, F.K., 2000. Molecular modeling of mammalian cytochrome P450s. Cell and Molecular Life Sciences 57, 487–499.

Daston, G.P., Baines, D., Yonker, J.E., 1991. Chick embryo retinal cell cultures as a screen for developmental toxicity. Toxicology and Applied Pharmacology 109, 352–366.

de Lange, J., van Eck, P., Elliott, G.R., de Kort, W.L., Wolthuis, O.L., 1992. The isolated blood perfused pig ear: an inexpensive and animal-saving model for skin penetration studies. Journal of Pharmacological and Toxicological Methods 27, 71–77.

Deaton, M.A., Bowman, P.D., Jones, G.P., Powanda, M.C., 1990. Stress protein synthesis in human keratinocytes treated with sodium arsentie, phenyldichloroarsine and nitrogen mustard. Fundamental and Applied Toxicology 14, 471–476.

Decker, T., Lohmann-Matthes, M.L., 1988. A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. Journal of Immunological Methods 15, 61–69.

DeJongh, J., Forsby, A., Houston, J.B., Beckmann, M., Combes, R., Blaauboer, B.J., 1999a. An integrated approach to the prediction of systemic toxicity using computer-based biokinetic models and biological *in vitro* test methode: overview of a prevalidation study based on the ECITTS project. Toxicology in Vitro 13, 549–554.

DeJongh, J., Nordin-Andersson, M., Ploeger, B., Forsby, A., 1999b. Estimation of systemic toxicity of acrylamide by integration of in vitro toxicity data with kinetic simulations. Toxicology and Applied Pharmacology 158, 261–268.

DeJongh, J., Verhaar, H.J., Hermens, J.L., 1997. A quantitative property-property relationship (QPPR) approach to estimate *in vitro* tissue-blood partition coefficients of organic chemicals in rats and humans. Archives of Toxicology 72, 17–25.

DeJongh, J., Verhaar, H.J.M., Hermens, J.L.M., 1998. Role of kinetics in acute lethality of nonreactive volatile organic compounds (VOCs). Toxicological Sciences 45, 26–32.

DeRisi, J., Penlans, L., Brown, P.O., et al., 1996. Use of cDNA microarray to analys gene expression patterns in human cancer. Nature Genetics 14, 457–460.

Diener, W., Gundert-Remy, U., 1999. Challenge for the successful prediction of drug metabolism in vivo from studies in vitro: Simulation of variability. In: Boobis, A.R., Kremers, P., Pelkonen, O., Pithan, K. (Eds.), European Symposium on Prediction of Drug Metabolism in Man: Progress and Problems. Office for Official Publications of the European Communities, Luxembourg , pp. 247–264.

Dierickx, P.J., 1989. Cytotoxicity testing of 114 compounds by the determination of the protein content in HepG2 cell cultures. Toxicology in Vitro 3, 189–193.

Dilworth, C., Hamilton, G.A., George, E., Timbrell, J.A., 2000. The use of liver spheriods as an in vitro model for studying inductions of the stress response as a marker for chemical toxicity. Toxicology in Vitro 14, 169–176.

Dilworth, C., Timbrell, J.A., 1998. An investigation into the sensitivity of heat shock proteins as markers of cellular damage: a comparative study of hydrazine and cadmium chloride in primary rat heoatocyte cultures. Biomarkers 3, 177–190.

Djuric, Z., Heilbrun, L.K., Reading, B.A., Boomer, A., Valerliote, F.A., Martino, S., 1991. Effects of a low-fat diet on levels of oxidative damage to DNA in human peripheral nucleated blood cells. Journal of the National Cancer Institute 83, 766–769.

Donati, Y.R.A., Slosman, D.O., Polla, P.S., 1990. Oxidative injury and the heat shock response. Biochemical Pharmacology 40, 2571–2577.

Duggan, D.J., Bittner, M., Chen, Y., Meltzer, P., Trent, J.M., 1999. Expression profiling using cDNA microarrays. Nature Genetics 21, 10–14.

Duthie, S.J., Collins, A.R., 1997. The influence of cell growth, detoxifying enzymes and DNA repair on hydrogen peroxide-mediated DNA damage (measured using the comet assay) in human cells. Free Radical Biology and Medicine 22, 717–724.

Duthie, S.J., Ma, A., Ross, M.A., Collins, A.R., 1996. Antioxidant supplementation decreases oxidative DNA damage in human lymphocytes. Cancer Research 56, 1291–1295.

Dybing, E., Doe, J., Groten, J., Kleiner, J., O'Brien, J., Renwick, A.G., Schlatter, J., Steinberg, P., Tritscher, A., Walker, R., Younes, M., 2002. Hazard characterisation of chemicals in food and diet: dose–response, mechanisms, and extrapolation issues. Food and Chemical Toxicology 40, 237–282.

Eads, C.A., Danenberg, K.D., Kawakami, K., Saltz, L.B., Blake, C., Shibata, D., Danenberg, P.V., Laird, P.W., 2000. MethyLight: a high-throughput assay to meassure DNA methylation. Nucleic Acids Research 28, E32.

EC, 24-11-1986. Council Directive 86/609/EEC of 24 November 1986.

Edler L., Poirier K, Dourson M., Kleiner J., Mileson B., Nordmann H., Renwick A., Slob W., Walton K., Würtzen G., 2002. Mathematical modeling and quantitative methods. Food and Chemical Toxicology 40, 283–326.

Eisen, M.B., Spellman, P.T., Brown, P.O., Botstein, D., 1998. Cluster analysis and display of genome-wide expression patters. Proceedings of the National Academy of Sciences of the U.S.A. 95, 14863–14868.

Elias, J.M., 1997. Cell proliferation indexes: a biomarker in solid tumors. Histochemistry 72, 78–85.

Fang, J.L., Vaca, C.E., Valsta, L.M., Mutanen, M., 1997. Determination of DNA adducts of malonaldehyde in humans: effects of dietary fatty acid composition. Carcinogenesis 17, 1033–1040.

FAO/WHO, 2001. Evaluation of Allergenicity of Genetically Modified Foods. Report of a Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology, 22–25 January 2001. Food and Agriculture Organisation of the United Nations (FAO), Rome.

Farber, J.L., 1990. The role of calcium in lethal cell injury. Chemical Research in Toxicology 3, 503–508.

FDA, 1982. Toxicological Principles for the Safety Assessment of Direct Food Additives and Color Additives Used in Food. Food and Drug Administration, Washington, DC.

Feig, D.I., Reid, T.M., Loeb, L.A., 1994. Reactive oxygen species in tumorigenesis. Cancer Research 54 (Suppl. 7), 1890s–1894s.

Fenech, M., Rinaldi, J., 1995. A comparison of lymphocyte micronuclei and plasma micronutrients in vegetarians and non-vegetarians. Carcinogenesis 16, 223–230.

Fenech, M., Stockley, C., Aitken, C., 1997. Moderate wine consumption protects against hydrogen peroxide-induced DNA damage. Mutagenesis 12, 289–296.

Fernandez-Metzler, C.L., Owens, K.G., Baillie, T.A., King, R.C., 1999. Rapid liquid Chromatography with tandem mass spectrometry-based screening procedures for studies on the biotransformation of drug candidates. Drug Metabolism and Disposition 27, 32–40.

Fiserova-Bergerova, V., Diaz, M.L., 1986. Determination and prediction of tissue-gas partition coefficients. International Archives of Occupational and Environmental Health 58, 75–87.

Flint, O.P., 1993. In vitro tests for teratogens:desirable endpoints, test batteries and current status of me micromass teratogen test. Reproductive Toxicology 7 (Suppl. 1), 103–111.

Fodor, P.A., Rava, R.P., Huang, X.C., Pease, A.C., Holmes, C.P., Adams, C.L., 1993. Nature 364, 555–556.

Franke, H., Galla, H., Beuckmann, C.T., 2000. Primary cultures of brain microvessel endithelial cells: a valid and flexible model to study drug transport through the blood-brain barrier in vitro. Brain Research Protocols 5, 248–256.

Friend, Dr., 1992. In vitro skin permeation techniques. Journal of Controlled Release 18, 235–248.

Fuglsang, G.M., Adsen, C., Saval, P., Osterballe, O., 1993. Prevalence of intolerance to food additives among Danish school children. Pediatric Allergy and Immunology 4, 123–129.

Gargas, M.L., Burgess, R.J., Voisard, D.E., Cason, G.H., Andersen, M.E., 1989. Partition coefficients of low-molecular-weight volatile chemicals in various liquids and tissues. Toxicology and Applied Pharmacology 98, 87–99.

Gargas, M.L., Seyboldt, P.G., Andersen, M.E., 1988. Modeling the tissue solubilities and metabolic rate constant (Vmax) of halogenated methanes, ethanes and ethylenes. Toxicology Letters 43, 235–256.

Garret, N.E., Campbell, J.A.H., Stack, H.F., Waters, M.D., Lewtas, J., 1981. The utilization of rabbit alveolar macrophage and Chinese hamster ovary cells for evaluation of the toxicity of particulate materials. Environmental Research 24, 324–332.

Gearhart, J.M., Jepson, G.W., Clewell, H.J., Andersen, M.E., Conolly, R., 1990. Physiologycally based pharmacokinetic and pharmacodynamic model for the inhibition of acetylcholinesterase by diisopropylfluorophosphate. Applied Pharmacology 106, 295–310.

Genschow, E., Scholz, G., Brown, N., Piersma, A.H., Brady, M., Clemann, N., Huusjkonen, H., Paillard, F., Bremer, S., Beker, K., Spielmann, H., 2000. Development of prediction models for three in vitro embryotoxicity tests in an ECVAM validation study. In Vitro and Molecular Toxicology 13, 51–65.

Gibson, D.P., Aardema, M.J., Kerckaert, G.A., Carr, G.J., Brauninger, R.M., LeBoeuf, R.A., 1995. Detection of aneuploidy-inducing carcinogens in the Syrian hamster embryo (SHE) cell transformation assay. Mutation Research 343, 7–24.

Glinghammer, B., Holmberg, K., Rafter, J., 1999. Effects of colonic luminal components on AP-1 dependent gene transcription in cultured human colon carcinoma cells. Carcinogenesis 20, 969–976.

Gocke, E., et al., 1999. Genotoxicity testing of biotechnology-derived products. Report of a GUM task force. Mutation Research 436, 137–156.

Goebel, C., Kubicka-Muranyi, M., Tonn, T., Gonzalez, J., Gleichmann, E., 1995. Phagocytes render chemicals immunogenic: oxidation of gold(I) to the T cell-sensitizing gold(III) metabolite generated by mononuclear phagocytes. Archives of Toxicology 69, 450–459.

Goering, P.L., Fisher, B.R., Kish, C.L., 1993. Stress protein synthesis induced in rat liver by cadmium precedes hepatotoxicity. Toxicology and Applied Pharmacology 122, 139–148.

Gonzalez, M.F., Shiraishi, K., Hisanga, K., Sagar, S.M., Mandabach, M., Sharp, F.R., 1989. Heat shock proteins as markers of neural injury. Molecular Brain Research 6, 93–100.

Graves, D.J., 1999. Powerful tools for genetic analysis come of age. Trends in Biotechnology 17, 127–134.

Griem, P., Panthel, K., Kalbacher, H., Gleichmann, E., 1996. Alteration of a model antigen by Au(III) leads to T cell sensitization to cryptic peptides. European Journal of Immunology 26, 279–287.

Gulumian, M., 1999. The role of oxidative stress in diseases caused by mineral dusts and fibres: current status and future of prophylaxis and treatment. Molecular and Cellular Biochemistry 196, 69–77.

Hanley, A.B., McBride, J., Oehlschläger, S., Opara, E., 1999. Use of a flow cell bioreactor as a chronic toxicity model system. Toxicology in Vitro 13, 847–851.

Hansen, D.K., Anson, J.F., Hinson, W.G., Pipkin, J.L., 1988. Phenytoin-induced stress protein synthesis in mouse embryonic tissue. Proceedings of the Society for Experimental Biology and Medicine 189, 136–140.

Hard, C.C., Rodgers, I.S., Baetcke, K.P., Richards, W.L., McGaughy, R.E., Valcovic, L.R., 1993. Hazard evaluation of chemicals that cause accumulation of alpha$_{2u}$-globulin, hyaline droplet nephropathy, and tubule neoplasia in the kidney of male rats. Environmental Health Perspectives 99, 313–349.

Harman, A.W., Maxwell, M.J., 1995. An evaluation of the role of calcium in cell injury. Annual Review of Pharmacology and Toxicology 35, 129–144.

Hartung, T., Gstraunthaler, G., 2000. The standardisation of cell culture practices. In: Balls, M., Van Zeller, A.-M., Halder, M.E. (Eds.), Progress in the Reduction, Refinement and Replacement of Animal Experiments. Elsevier, Amsterdam, pp. 1655–1658.

Haseltine, W.A., Franklin, W., Lippke, J.A., 1983. New methods for detection of low levels of DNA damage in human populations. Environmental Health Perspectives 48, 29–41.

Hasmall, S.C., Roberts, R.A., 1999. The perturbation of apoptosis and mitosis by drugs and xenobiotics. Pharmacology and Therapeutics 82, 63–70.

Hauser, C., Katz, S.I., 1988. Activation and expansion of hapten- and protein-specific T helper cells from nonsensitized mice. Proceedings of the National Academy of Sciences of the U.S.A. 85, 5625–5628.

Hayashi, Y., 1992. Overview of genotoxic carcinogens and non-genotoxic carcinogens. Experimental Toxicology and Pathology 44, 465–471.

Hayatsu, H., Hayatsu, T., Ohara, Y., 1985. Mutagenicity of human urine caused by ingestion of fried ground beef. Japanese Journal of Cancer Research 76, 445–448.

Heid, C.A., Stevens, J., Livak, K.A., Williams, P.M., 1996. Real Time Quantitative PCR. Genome Research 6, 986–994.

Helenius, A., Trombetta, E.S., Hebert, D.N., Simons, J.F., 1997. Calnexin, calreticulin and the folding of glycoproteins. Trends in Cell Biology 7, 193–200.

Henderson, B.E., Ross, R.K., Pike, M.C., 1991. Toward the primary prevention of cancer. Science 254, 1131–1138.

Hertel, S., Heinz, F., Vogel, M., 2000. Hydrolysis of low-molecular-weight oligosaccharides and oligosaccaride alditols by pig intestinal/isomaltase and glucosidase/maltase. Carbohydrate Research 326, 359–367.

Heuer, J., Graeber, I.M., Pohl, I., Spielmann, H., 1994. An in vitro embryotoxicity assay using the differentiation of embryonic mouse stem cells into haematopoietic cells. In Vitro Toxicology 8, 585–587.

Hildebrand, R.L., Andersen, M.E., Jenkins, L.J., 1981. Prediction of in vivo kinetic constants from kinetic constants measured in vitro. Fundamental and Applied Toxicology 1, 403–409.

Hojo, M., Morimoto, T., Maluccio, M., Asano, T., Morimoto, K., Lagman, M., Shimbo, T., Suthanthiran, M., 1999. Cyclosporine induces cancer progression by a cellautonomous mechanism. Nature 397, 530–534.

Holden, H.T., Lichter, W., Sigel, M., 1973. Quantitative methods for measuring the cell growth and death. In: Kruse, P., Patterson, M.K. (Eds.), Tissue Culture: Methods and Applications. Academic Press, New York , pp. 408–411.

Hosokawa, N., Hirayoshi, K., Kudo, H., Takechi, H., Aoike, A., Kawai, K., Nagata, K., 1992. Inhibition of the activation of heat shock factor in vivo and in vitro by flavonoids. Molecular and Cellular Biology 12, 3490–3498.

Houston, J.B., Carlile, D.J., 1997. Prediction of hepatic clearance from microsomes, hepatocytes and liver slices. Drug Metabolism Reviews 29, 891–922.

IARC, 1987. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Sodium Ortho-pheylphenate. Overall Evaluations of Carcinogencicity: An Updating of IARC Monographs Volumes 1 to 42 (Suppl. 7). International Agency for Research on Cancer, Lyon.

IARC, 1988. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Vol. 44. Alcohol Drinking. International Agency for Research on Cancer, Lyon.

IARC, 1997. Polychlorinated dibenzo-para-dioxins. In: IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Vol. 69. Polychlorinated Dibenzo-dioxins and Polychlorinated Dibenzofurans. International Agency for Research on Cancer, Lyon.

IARC, 1999. Short- and Medium-term Carcinogenicity Tests, Mutation Analysis, and Multistage Models in Risk Identification. IARC Scientific Publications no. 146. International Agency for Research on Cancer, Lyon.

ICCVAM, 1997a. Validation and Regulatory Acceptance of Toxicological Test Methods. A Report of the Ad Hoc Interagency Coordinating Committee on the Validation of Alternative Methods. NIEHS, Research Triangle Park, NC.

ICCVAM, 1997b. Validation and Regulatory Acceptance of Toxicological Test Methods. A Report of the Ad Hoc Interagency Coordinating Committee on the Validation of Alternative Methods. NIEHS, Research Triangle Park, NC.

IPCS, 1994. Environmental Health Criteria. Volume 16: Chloroform. World Health Organization, Geneva.

Ito, K., Iwatsubo, T., Kanamitsu, S., Nakajima, Y., Sugiyama, Y., 1998. Quantitative prediction of in vivo drug clearance and drug interactions from in vitro data on metabolism, together with binding and transport. Annual Review of Pharmacology and Toxicology 38, 461–499.

Ito, K., Kusuhara, H., Sugiyama, Y., 1999. Effects of intesinal CYP3A4 and P-glycoprotein on oral drug absorption—theoretical approach. Pharmaceutical Research 16, 225–231.

James, N.H., Roberts, R.A., 1996. Species differences in response to peroxisome proliferators correlate in vitro with induction of DNA synthesis rather than suppression of apoptosis. Carcinogenesis 17, 1623–1632.

Jelinek, R., 1977. The chick embryotoxicity screening test (CHEST). In: Neubert, D., Merker, H.J., Kwasigroch, T.E. (Eds.), Methods in Prenatal Toxicology. Georg Thieme Verlag, Stuttgart, pp. 381–386.

Jelinek, R., Peterka, M., Rychter, Z., 1985. Chick embryotoxicity screening test–130 substances tested. Indian Journal of Experimental Biology 23, 588–595.

Jenkins, G.J., Suzen, H.S., Gueiro, R.A., Parry, J.M., 1999. The restriction site mutation assay: a review of the methodology development and the current status of the technique. Mutagenesis 14, 439–448.

Jepson, G.W., Hoover, D.K., Black, R.K., McCafferty, J.D., Mahle, D.A., Gearhart, J.M., 1994. A partition coefficient determination method for nonvolatile chemicals in biological tissues. Fundamental and Applied Toxicology 22, 519–524.

Kao, J., Patterson, K., Hall, J., 1985. Skin penetration and metabolism of topically applied chemicalss in six mammalian species, including man: an in vitro study with benzo(a)pyrene and testosterone. Toxicology and Applied Pharmacology 81, 502–516.

Kappers, W.A., van Och, F.M., de Groene, E.M., Horbach, G.J., 2000. Comparison of three different in vitro mutation assays used for the investigation of cytochrome P450-mediated mutagenicity of nitro-polycyclic aromatic hydrocarbons. Mutation Research 466, 143–159.

Karnes Jr., W.E., Walsh, J.H., 1990. The gastrin hypothesis. Implications for antisecretory drug selection. Journal of Clinical Gastroenterology 12 (Suppl. 2), 7–12.

Kass, G.E.N., Orreniun, S., 1998. Calcium signalling and cytotoxicity. Environmental Health Perspectives 107 (Suppl. 1), 25–35.

Kaufman, R.J., 1999. Stress signalling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. Genes Development 13, 1211–1233.

Kedderis, G.L., Carfagna, M.A., Held, S.D., Batra, R., Murphy, J.E., Gargas, M.L., 1993. Kinetic analysis of furan biotransformation by F-344 rats in vivo and in vitro. Toxicology and Applied Pharmacology 123, 274–282.

Kim, Y.I., Mason, J.B., 1996. Nutrition chemoprevention of gastrointestinal cancers: a critical review. Nutrition Reviews 54, 259–279.

Klaassen, C.D., Liu, J., 1998. Induction of metallothionein as an adaptive mechanism affecting the magnitude and progreeion of toxicological injury. Environmental Health Perspectives 106, 297–300.

Klaunig, J.E., Xu, Y., Isenberg, J.S., Bachowski, S., Kolaja, K.L., Jiang, J., Stevenson, D.E., Walborg Jr., E.F., 1998. The role of oxidative stress in chemical carcinogenesis. Environmental Health Perspectives 106 (Suppl. 1), 295.

Klein, C.B., Costa, M., 1997. DNA methylation, heterochromatin and epigenetic carcinogens. Mutation Research 386, 163–180.

Klein, G., Klein, E., 1984. Commentary: oncogene activation and tumor suppression. Carcinogenesis 5, 3362–3364.

Kohler, J., Hartmann, U., Grimm, R., Pflugfelder, U., Weltzien, H.U., 1997. Carrier-independent hapten recognition and promiscuous MHC restriction by CD4 T cells induced by trinitrophenylated peptides. Journal of Immunology 158, 591–597.

Kondoh, N., Wakatsuki, T., Ryo, A., Hada, A., Aihara, T., Horiuchi, S., Goseki, N., Matsubara, O., Takenaki, K., Shichita, M., Tanaka, K., Shuda, M., Yamamoto, M., 1999. Identification and characterization of genes associated with human hepatocellular carcinogenesis. Cancer Research 59, 4990–4996.

Korfmacher, W.A., Palmer, C.A., Mardo, C., Dunn-Meynell, K., Grotz, D., Cox, K., Lin, C.C., Elicone, C., Liu, C., Duchoslav, E., 1999. Development of an automated mass spectrometry system for the quantitative analysis of liver microsomal incubation samples: a tool for rapid screening of new compounds for metabolic stability. Rapid Communications in Mass Spectrometry 13, 901–907.

Kramer, P.J., 1998. Genetic toxicology. Journal of Pharmacy and Pharmacology 50, 395–405.

Kroes, R., Galli, C., Munro, I., Schilter, B., Tran, L.A., Walker, R., Würzen, G., 2000. Threshold of toxicological concern for chemical substances present in the diet: a practical tool for assessing the need for toxicity testing. Food and Chemical Toxicology 38, 255–312.

Kroes, R., Müller, D., Lambe, J., Löwik, M.R.H., van Klaveren, J., Kleiner, J., Massey, R., Mayer, S., Urieta, I., Verger, P., Visconti, A., 2002. Assessment of intake from the diet. Food and Chemical Toxicology 40, 327–385..

Kucera, P., Cano, E., Honegger, P., Schilter, B., Zijstra, J.A., Schmid, B., 1993. Validation of whole chick embryo cultures, whole rat embryo cultures and aggregating embryonic brain cell cultures using six pairs of coded compounds. Toxicology in Vitro 7, 785–798.

Lave, T., Coassolo, P., Reigner, B., 1999. Prediction of hepatic metabolic clearance based on interspecies allometric scaling techniques and in vitro–in vivo correlations. Clinical Pharmacokinetics 36, 211–231.

LeCluyse, E., Madan, A., Hamilton, G., Carroll, K., DeHaan, R.P.A., 2000. Expression and regulation of cytochrome P450 enzymes in primary cultures of human hepatocytes. Journal of Biochemistry and Molecular Toxicology 14, 177–188.

Lee, A.S., 1992. Mammalian stress response: inductions of the glucose-regulated protein family. Current Opinion in Cell Biology 4, 267–273.

Lennernas, H., 1998. Human intestinal permeability. Journal of Pharmaceutical Sciences 87, 410.

Liang, P., Pardee, A.B., 1992. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. Science 257, 967–971.

Loeb, K.R., Loeb, L.A., 2000. Significance of multiple mutations in cancer. Carcinogenesis 21, 379–385.

Low-Friedrich, I., von Bredow, F., Schoeppe, W., 1991. A cell culture assay for the detection of cardiotoxicity. Journal of Pharmacological Methods 25, 133–145.

Machlin, L.J., 1995. Critical assessment of the epidemiological data concerning the impact of antioxidant nutrients on cancer and cardiovascular disease. Critical Reviews in Food Science and Nutrition 35, 41–50.

Mao, L., Lee, D.J., Tockman, M.S., Erozan, Y.S., Askin, F.B., Sidransky, D., 1994. Microsatellite alterations as clonal markers for the detection of human cancer. Proceedings of the National Academy of Sciences of the U.S.A. 91, 9871–9875.

Marie, I.J., Dalet, C., Blanchard, J.M., Astre, C., Szawlowski, A., Saint Aubert, B., Joyeux, H., Maurel, P., 1988. Inhibition of cytochrome P-450p (P450IIIA1) gene expression during liver regeneration from two-thirds hepatectomy in the rat. Biochemical Pharmacology 37, 3515–3521.

Marsman, D.S., Barrett, J.C., 1994. Apoptosis and chemical carcinogenesis. Risk Analysis 14, 321–326.

Martin, L.D., Krunkosky, T.M., Dye, J.A., Fischer, B.M., Jiang, N.F., Rochelle, L.G., Akley, N.J., Dreher, K.L., Adler, K.B., 1997. The role of reactive oxygen and nitrogen species in the response of airway epithelium to particulates. Environmental Health Perspectives 105 (Suppl. 5) 1301–1307.

McBurney, M.I., Thompson, L.U., 1989. Effect of human faecal donor on in vitro fermentation variables. Scandinavian Journal of Gastroenterology 24, 359–367.

McClain, R.M., 1989. The significance of hepatic microsomal enzyme induction and altered thyroid function in rats: implications for thyroid gland neoplasia. Toxicologic Pathology 17, 294–306.

McConkey, D.J., Orrenius, S., 1997. The role of calcium in the regulation of apoptosis. Biophysical Research Communications 239, 357–366.

McCusker, K., 1992. Mechanism of respiratory tissue injury from cigarette smoking. American Journal of Medicine 93, 18–21.

McDougal, J.N., Jepson, G.W., Clewell, H.J., Gargas, M.L., Andersen, M.E., 1990. Dermal absorption of organic chemical vapours in rats and humans. Fundamental and Applied Toxicology 14, 299–308.

McGregor, D.B., Partensky, C., Wilbourn, J., Rice, J.M., 1998. An IARC evaluation of polychlorinated dibenzo-p-dioxins and polychloinated dibenzofurans as risk factors in human carcinogenesis. Environmental Health Perspectives 106 (Suppl. 2), 755–760.

Menzel, D.B., Rasmussen, R.E., Lee, E., Meacher, D.M., Said, B., Hamadeh, H., Vargas, M., Greene, H., Roth, R.N., 1998. Human lymphocyte heme oxygenase 1 as a response biomarker to inorganic arsenic. Biochemical and Biophysical Research Communications 250, 653–656.

Metcalfe, D.D., Astwood, J.D., Townsend, R., Sampson, H.A., Taylor, S.L., Fuchs, R.L., 1996. Assessment of the allergenic potential of foods derived from genetically engineered crop plants. Critical Reviews in Food Science and Nutrition 36 (Suppl), S165–S186.

Minamoto, T., Mai, M., Ronai, Z., 1999. Environmental factors as regulators and effectors of multistep carcinogenesis. Carcinogenesis 20, 519–527.

Muller, L., Kikuchi, Y., Probst, G., Schechtman, L., Shimada, H., Sofuni, T., Tweats, D., 1999. ICH-harmonised guidances on genotoxicity testing of pharmaceuticals: evolution, reasoning and impact. Mutation Research 436, 195–225.

Mummery, C.L., van der Brink, C.E., van der Saag, P.T., De Laat, S.W., 1984. A short term screening test for teratogens using differentiating neuroblastoma cells in vitro. Teratology 29, 271–279.

Nakajima, M., Takeuchi, T., Takeshita, T., Morimoto, K., 1996. 8-Hydroxydeoxyguanosine in human leukocyte DNA and daily health practice factors: Effects of individual alcohol sensitivity. Environmental Health Perspectives 104, 1336–1338.

Nau, H., 1990. Pharmacokinetic aspects of in vitro teratogenicity studies: comparison to in vivo. In: Kimmel, G.L., Kochhar, D.M. (Eds.), In Vitro Methods in Developmental Toxicology: Use in Defining Mechanisms and Risk Parameters. CRC Press, Boca Raton, FL, pp. 29–43.

Nguyen-Ba, G., Vasseur, P., 1999. Epigenetic events during the process of cell transformation induced by carcinogens (review). Oncology Reports 6, 925–932.

Nicotera, P., Bellomo, G., Orrenius, S., 1990. The role of $Ca^{2+}$ in cell killing. Chemical Research in Toxicology 3, 484–494.

Nielsen, F., Mikkelsen, B.B., Nielsen, J.B., Andersen, H.R., Grandjean, P., 1997. Plasma malondialdehyde as biomarker for oxidative stress: reference interval and effects of life-style factors. Clinical Chemistry 43, 1209–1214.

Ninomiya H., Kishida K., Ohno Y., Tsurumi K., Eto K., 1993. Effects of trypan blue on rat and rabbit embryos cultured *in vitro*. Toxicology in Vitro 7, 717.

Nishikawa, J., Saito, K., Goto, J., Dakeyama, F., Matsuo, M., Nishihara, T., 1999. New screening methods for chemicals with hormonal activities using interaction of nuclear hormone receptor with coactivator. Toxicology and Applied Pharmacology 154, 76–83.

North-Root, H., Yackovitch, F., Demetrulias, J., Gacula, M., Heinz, J.E., 1982. Evaluation of an in vitro cell toxicity using rabbit corneal cells to predict the eye-irritation potential of surfactants. Toxicology Letters 14, 107–214.

Nuwaysir, E.F., Bittner, M., Trent, J., Barrett, J.C., Afshari, C.A., 1999. Microarrays and Toxicology: The Advent of Toxicogenomics. Molecular Carcinogenesis 24, 153–159.

O'Farrell, P.H., 1975. High resolution two-dimensional electrophoresis of proteins. Journal of Biological Chemistry 250, 4007–4021.

Obach, R.S., Baxter, J.G., Liston, T.E., Silber, B.M., Jones, B.C., MacIntyre, F., Rance, D.J., Wastall, P., 1997. The prediction of human pharmacokinetic parameters from preclinical and in vitro meatbolism data. Journal of Pharmacology and Experimental Therapy 283, 46–58.

OECD, 1996. Final Report of the OECD Workshop on Harmonization of Validation and Acceptance Criteria for Alternative Toxicological Tests Methods. Organisation for Economic Cooperation and Development, Paris.

Olden, K., 1994. Mutagen hypersensitivity as a biomarker of genetic predisposition to carcinogenesis. Journal of the National Cancer Institute 86, 1660–1661.

Ortolani, C., Ispano, M., Scibilia, J., Pastorello, E.A., 2001. Introducing chemists to food allergy. Allergy 56 (Suppl), 5–8.

Pacifici, G.M., Viani, A., 1992. Methods of determining plasma and tissue binding of drugs. Clinical Pharmacokinetics 23, 449–468.

Padovan, E., von Greyerz, S., Pichler, W.J., Weltzien, H.U., 1999. Antigen-dependent and-independent IFN-gamma modulation by penicillins. Journal of Immunology 162, 1171–1177.

Pepe, M.S., Etzioni, R., Feng, Z., Potter, J.D., Thompson, M.L., Thornquist, M., Winget, M., Yasui, Y., 2001. Phases of biomarker development for early detection of cancer. Journal of the National Cancer Institute 93, 1054–1061.

Perbellini, L., Brugnone, F., Caretta, D., Maranelli, G., 1985. Partition Coefficients of some industrial aliphatic hydrocarbons (C5-C7) in blood and human tissues. British Journal of Industrial Medicine 42, 162–167.

Perera, F.P., 1996. Molecular epidemiology: insights into cancer susceptibility, risk assessment, and prevention. Journal of the National Cancer Institute 88, 509.

Perera, F.P., Whyatt, R.M., 1994. Biomarkers and molecular epidemiology in mutation/cancer research. Mutation Research 313, 117–129.

Pfaller, W., Balls, M., Clothier, R., Coecke, S., Dierickx, P., Ekwall, B., Hanley, B.A., Hartung, T., Prieto, P., Ryan, M.P., Gschmuck, G., Sladowski, D., Vericat, J.C., Wendes, A., Wolf, A., Zimmer, J., 2001. Novel in vitro methods for long-term toxicity testing. ATLA 29, 393–426.

Piersma, A.H., Attenon, P., Bechter, R., Govers, M.J.A.P., Krafft, N., Schmid, B.P., Stadler, J., Verhoef, A., Verseil, C., 1995. Interlaboratory evaluation of embryotoxicity in the postimplantation rat embryo culture. Reproductive Toxicology 9, 275–280.

Pipkin, J.L., Anson, J.F., Hinson, W.G., Burns, E.R., Casciano, D.A., 1988. Comparative immunochemical and structural similarities of five stress proteins from various tissue types. Comparative Biochemistry and Physiology B89, 43–50.

Pitot, H.C., 1993. The molecular biology of carcinogenesis. Cancer 72, 962–970.

Pool-Zobel, B.L., Bub, A., Müller, H., Wollowski, I., Rechkemmer, G., 1997. Consumption of vegetables reduces genetic damage in humans: first results of an intervention trial with carotenoid-rich foods. Carcinogenesis 18, 1847–1850.

Pool-Zobel, B.L., Leucht, U., 1997. Induction of DNA damage in human colon cells derived from biopsies by suggested risk factors of colon cancer. Mutation Research 375, 105–116.

Pool-Zobel, B.L., Lotzmann, N., Knoll, M., Kuchenmeister, F., Lambertz, R., Leucht, U., Schröder, H.G., Schmezer, P., 1994. Detection of genotoxic effects in human gastric and nasal mucosa cells isolated from biopsy samples. Environmental and Molecular Mutagenesis 24, 23–45.

Pratt, R.M., Grove, R.I., Willis, W.D., 1982. Prescreening for environmental teratogens using cultured mesenchymal cells from the human embryonic palate. Teratogenesis, Carcinogenesis, and Mutagenesis 2, 313–318.

Preston-Martin, S., Pike, M.C., Ross, P.K., Jones, P.A., Hederson, B.E., 1990. Increased cell division as a cause of human cancer. Cancer Research 50, 7415–7421.

Reed, D.J., 1990. Review of the current status of calcium and thiols in cellular injury. Chemical Research in Toxicology 3, 495–502.

Rensing, S.A., Maier, U.-G., 1994. Phylogenetic analysis of the stress-70 protein family. Journal of Molecular Evolution 39, 80–86.

Reuber, M.D., 1989. Carcinogenity of captan. Journal of Environmental Pathology, Toxicology and Oncology 9, 127–143.

Rieger, M.A., Parlesak, A., Pool-Zobel, B.L., Rechkemmer, G., Bode, C., 1999. Diets high in fat and meat, but low in fiber increase the genotoxic potential of fecal water. Carcinogenesis 20, 2317–2326.

Roberts, D.W., Williams, D.L., 1982. The derivation of quantitative correlations between skin sensitisation and physiko-chemical parameters for alkylating agents, and their application to experimental data for sultones. Journal of Theoretical Biology 99, 807–825.

Rockett, J.C., Dix, D.J., 1999. Application of DNA arrays to toxicology. Environmental Health Perspectives 107, 685.

Roe, F.J., 1998. A brief history of the use of laboratory animals for the prediction of carcinogenic risk for man with a note on needs for the future. Experimental Toxicology and Pathology 50, 271–276.

Rohwedel, J., Maltsev, V., Bober, E., Arnold, H.H., Hescheler, J., Wobus, A.M., 1994. Muscle cell differentiation of embryonic stem cells reflects myogenesis in vivo: developmentally regulated expression of myogenic determination genes and functional expression of ionic currents. Developmental Biology 164, 87–101.

Roses, A.D., 2000. Pharmacogenetics and the practice of medicine. Nature 405, 857–865.

Rumney, C.J., Rowland, I.R., 1992. In vivo and in vitro models of the human colonic flora. Critical Reviews in Food Science and Nutrition 31, 299-331.

Rustemeyer, T., de Groot, J., von Blomberg, B.M., Frosch, P.J., Scheper, R.J., 1998. Crossreactivity patterns of contact-sensitizing methacrylates. Toxicology and Applied Pharmacology 148, 83–90.

Rustemeyer, T., De Ligter, S., von Blomberg, B.M., Frosch, P.J., Scheper, R.J., 1999. Human T lymphocyte priming in vitro by haptenated autologous dendritic cells. Clinical and Experimental Immunology 117, 209–216.

Sadler, T.W., Horton, W.E., Warner, C.W., 1982. Whole embryo culture: a screening technique for teratogens? Teratogenesis, Carcinogenesis, and Mutagenesis 2, 243–253.

Sato, A., Nakajima, T.A., 1979. A structure-activity relationship of some chlorinated hydrocarbons. Archives of Environmental Health 69–75.

Schena, M., Davis, R.W., Brown, P.O., 1995. Quantitative monitoring of gene-expression patterns with a complementary DNA microarray. Science 270, 467–470.

Schmid, B.P., 1985. Teratogenicity testing of new drugs with the postimplantation embryo culture system. In: Homburger, F. (Ed.), Concepts in Toxicology. Karger, Basel , pp. 46–57.

Schut, H.A.J., Shiverick, K.T., 1992. DNA adducts in humans as dosimeters of exposture to environmental, occupational, or dietary genotoxins. FASEB Journal 6, 2942–2951.

Segall, M.D., Payne, M.C., Ellis, S.W., Tucker, G.T., Eddershaw, P.J., 1999. First principles investigation of singly reduced cytochrome P450. Xenobiotica 29, 561–571.

Seibert, H., Balls, M., Fentem, J.H., Bianchi, V., Clothier, R.H., Dierickx, P.J., Ekwall, B., Garle, M.J., Gomez-Lechon, M.J., Gribaldo, L., Gülden, M., Liebsch, M., Rasmussen, E., Roguet, R., Shivrastava, R., Walum, E., 1996. Acute toxicity testing *in vitro* and the classification and labelling of chemivals. The report and recommendations of ECVAM workshop 16. ATLA 24, 499–510.

Seydel, J.K., Schaper, K.J., 1982. Quantitative structure-pharmacokinetic relationships and drug design. Pharmacology and Therapeutics 407–414.

Shi, X., Castranova, V., Halliwell, B., Vallyathan, V., 1998. Reactive oxygen species and silica-induced carcinogenesis. Journal of Toxicology and Environmental Health B. Critical Reviews 1, 181–197.

Shopsis, C., Sathe, S., 1984. Uridine uptake inhibition as a cytotoxicity test: correlations with the draize test. Toxicology 29, 195–206.

Sobels,F.H., 1980. Evaluating the mutagenic potential of chemicals. The minimal battery and extrapolation problems. Archives of Toxicology 46, 21-30.

Spielmann, H., 1998. Reproduction and development. Environmental Health Perspectives 106 (Suppl. 2), 571–576.

Spielmann, H., Balls, M., Dupuis, J., Pape, W.J.W., Pechovitch, G., deSilva, O., Holzhütter, H.G., Clothier, R., Desolle, P., Gerberick, F., Liebsch, M., Lovell, W.W., Maurer, T., Pfannenbecker, U., Potthast, J.M., Csato, M., Sladowski, D., Steiling, W., Brantom, P., 1998. EU/COLIPA "*In vitro* phototoxicity" validation study, results of phase II (blind trial), part 1: the 3T3 NRU phototoxicity test. Toxicology in Vitro 12, 305–327.

Spielmann, H., Liebsch, M., 2001. Lessons learned from validation of in vitro toxicity test: from failture to acceptence into regulatory practice. Toxicology in Vitro 15, 585–590.

Spielmann, H., Pohl, I., Doring, B., Moldenhauer, F., 1997. The embryonic stem cell test (EST): an in vitro embryotoxicity test using two permanent mouse cell lines. In Vitro Toxicology 10, 127–199.

Spielmann, H., Vogel, R., 1989. Unique role of studies on preimplantation embryos to understand mechanisms of embryotoxicity in early pregnancy. Critical Reviews in Toxicology 20, 51–64.

Steele, V.E., Morrissey, R.E, Elmore, E.L., Gurganus-Rocha, D., Wilkinson, B.P., Curren, R.D., Schmetter, B.S., Louie, A.T., Lamb, J.C., Yang, L.I.L., 1988. Evaluation of two in vitro assays to screen for potential development toxicants. Fundamental and Applied Toxicology 11, 673–684.

Stejskal, V.D.M., Olin, R.G., Forsbeck, M., 1986. The lymphocyte transformation test for diagnosis of drug-induced occupational allergy. Journal of Allergy and Clinical Immunology 77, 411–426.

Strom, S.C., Novicki, D.L., Novotny, A., Jirtle, R.L., Michalopoulos, G., 1983. Human hepatocyte-mediated mutagenesis and DNA repair activity. Carcinogenesis 4, 683–686.

Thelestam, M., Molby, R., 1976. Cytotoxic effects on the plasma membrane of human diploid fibroblasts, a comparative study of leakage tests. Medical Biology 54–65.

Thomas, R.S., Yang, R.S., Morgan, D.G., Moorman, M.P., Kermani, H.R., Sloane, R.A., O'Conner, R.W., Adkins Jr., B., Gargas, M.L., Andersen, M.E., 1996. PBPK modeling/Monte Carlo simulation of methylene chloride kinetic changes in mice in relation to age and acute, subchronic, and chronic inhalation exposure. Environmental Health Perspectives 104, 858–865.

Toftgard, R., Haaparanta, T., Eng, L., Halpert, J., 1986. Rat lung and liver microsomal cytochrome P450 isoenzymes involved in the hydroxylation of n-hexane. Biochemical Pharmacology 35, 3733–3738.

Trump, B.F., Berezesky, I.K., 1995. Calcium-mediated cell injury and cell death. FASEB Journal 9, 219–228.

Tucker, J.D., Eastmond, D.A., Littlefield, L.G., 1997. Cytogenetic end-points as biological dosimeters and predictors of risk in epidemiological studies. In: Tonniolo, P., Boffetta, P., Shuker, D.E.G., Rothman, N., Hulka, B., Pearce, N. (Eds.), Application of Biomarkers in Cancer Epidemiology. IARC Publications no. 142. International Agency for Research on Cancer, Lyon , pp. 185–200.

Umansky, R., 1966. The effect of cell population density on the developmental fate of reaggregating mouse limb bud mesenchyme. Developmental Biology 13, 31–56.

Vainio,H., Magee, P.N., McGregor, D.B., McMichael, A.J., 1992. Mechanisms of Carcinogenesis in Risk Identification. IARC Publications no. 116. International Agency for Research on Cancer, Lyon. pp. 615.

Valentin, I., Lhuguenot, J.C., Chagnon, M.C., 2000. Uridine uptake inhibition as a cytotoxicity test for a human hepatoma cell line (HepG2 cells): comparison with the neutral red assay. Biochimica et Biophysica Acta 794, 373–380.

van den Brandt, P., Voorrips, L., Hertz-Picciotto, I., Shuker, D., Boeing, H., Speijers, G., Guittard, C., Kleiner, J., Knowles, M., Wolk, A., Goldbohm, A., 2002. The contribution of epidemiology. Food and Chemical Toxicology 40, 387–424.

Velculescu, V.E., Zhang, L., Vogelstein, B., Kinzler, K.W., 1995. Serial analysis of gene expression. Science 270, 484–487.

Venturi, M., Hambly, R.J., Glinghammer, B., Rafter, J.J., Rowland, I.R., 1997. Genotoxic activity in human faecal water and the role of bile acids: a study using the alkaline comet assay. Carcinogenesis 18, 2353–2359.

Vorm, O., Mann, M.J., 1994. Improved mass accuracy in matrix-assisted laser desorption/ionization time-of-flight mass-spectrometry of peptides. Journal of the American Society for Mass Spectrometry 5, 955–958.

Wachsman, J.T., 1997. DNA methylation and the association between genetic and epigenetic changes: relation to carcinogenesis. Mutation Research 375, 1–8.

Walton, K., Walker, R., van de Sandt, J.J.M., Knapp, A.G.A.A., Kozianowski, G., Roberfroid, M.B., Schilter, B., 1999. The application of *in vitro* data in the derivation of the acceptable daily intake of food additives. Food and Chemical Toxicology 37, 1175–1197.

Walum, E., Balls, M., Bianchi, V., Blaauboer, B.J., Bolcsfoldi, G., Guillouzo, A., Moore, G.A., Odland, L., Reinhard, C., Spielmann, H., 1992. ECITTS: an integrated approach to the application of in vitro test systems to the hazard assessment of chemicals. ATLA 20, 406–428.

Watson, A., Mazumder, A., Stewart, M., Balasubramanian, S., 1998. Technology for microarray analysis of gene expression. Current Opinions in Biotechnology 9, 609–614.

Waxman, D.J., 1999. P450 gene induction by structurally diverse xenochemicals: central role of nuclear receptors CAR, PXR, and PPAR. Archives of Biochemistry and Biophysics 369, 11–23.

Werner, L., Legeais, J.M., Nagel, M.D., Renard, G., 1999. Neutral red assay of the cytotoxicity of fluorocarbon coated polymethylmethacrylate intraocular lenses in vitro. Journal of Biological Material Research 148, 814–819.

Whitten, P.L., Naftolin, F., 1998. Reproductive actions of phytoestrogens. Baillieres Clinical Endocrinology and Metabolism 12, 667–690.

Wild, C.P., Pisani, P., 1997. Carcinogen-DNA and carcinogen-protein adducts in molecular epidemiology. In: Tonniolo, P., Boffetta, P., Shuker, D.E.G., Rothman, N., Hulka, B., Pearce, N. (Eds.), Application of Biomarkers in Cancer Epidemiology. IARC Publications no. 142. International Agency for Research on Cancer, Lyon , pp. 143–158.

Williams, G.M., Whynser, J., 1996. Epigenetic carcinogens: evaluation and risk assessment. Experimental Toxicologic Pathology 48, 189–195.

Williams, K.L., Hochstrasser, D.F., 1997. Proteome Research: New Frontiers in Functional Genomics. Springer, Berlin.

Wilson, N., Scott, A., 1989. A double-blind assessment of additive intolerance in children using a 12 day challenge period at home. Clinical and Experimental Allergy 19, 267–272.

Wortelboer, H.M., deKruif, C.A., van Iersel, A.A.J., Falke, H.E., Noordhoek, J., Blaauboer, B.J., 1991. Comparison of cytochrome P450 isoenzyme profiles in rat liver and hepatocyte cultures. Biochemical Pharmacology 42, 381–390.

Wu, Y., Chen, J., Ohshima, H., Pignatelli, B., Boreham, J., Li, J., Campbell, T.C., Peto, R., Bartsch, H., 1993. Geographic association between urinary excretion of N-nitroso compounds and oesophageal cancer mortality in China. International Journal of Cancer 54, 713–719.

Yadollahi-Farsani, M., Gooderham, M.J., Davies, D.S., Boobis, A.R., 1996. Mutational spectra of the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) at the chinese hamster *hprt* locus. Carcinogenesis 17, 617–624.

Yamasaki, H., Ashby, J., Bignami, M., Jongen, W., Linnainmaa, K., Newbold, R.F., Nguyen-Ba, G., Parodi, S., Rivedal, E., Schiffmann, D., Simons, J.W., Vasseur, P., 1996. Nongenotoxic carcinogens: development of detection methods based on mechanisms: a European project. Mutation Research 353, 47–63.

Yang, R.S., Andersen, M.E., 1994. Pharmacokinetics. In: Hodgson, E., Levi, P.E. (Eds.), Introduction to Biochemical Toxicology. Appleton and Lange, Norwalk, VA.

Yang, R.S., Thomas, R.S., Gustafson, D.L., Campain, J., Benjamin, S.A., Verhaar, H.J., Mumtaz, M.M., 1998. Approaches to developing alternative and predictive toxicology based on PBPK/PD and QSAR modeling. Environmental Health Perspectives 106 (Suppl. 6), 1385–1393.

Yokozeki, H., Katayama, I., Nishioka, K., 1995. Experimental study for the development of an in vitro test for contact allergens. 1. Primary activation of hapten-specific T cells by hapten-conjugated epidermal cells. International Archives of Allergy and Immunology 106, 394–400.

Zeiger, E., 1994. Strategies and philosophies of genotoxicity testing. What is the question? Mutation Research 304, 309–314.

Zhao, N., Hashida, H., Takahashi, N., Misumi, Y., Sakaki, Y., 1995. High-density cDNA filter analysis: a novel approach for large-scale, quantitative analysis of gene expression. Gene 156, 207–213.

Zhou, D., Salnikow, K., Costa, M., 1998. Cap43, a novel gene specifically induced by Ni2+ compounds. Cancer Research 58, 2182–2189.