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Food and Chemical Toxicology 40 (2002) 237–282

Food and Chemical Toxicology

www.elsevier.com/locate/foodchemtox

Hazard characterisation of chemicals in food and diet: dose response, mechanisms and extrapolation issues☆

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Summary

Hazard characterisation of low molecular weight chemicals in food and diet generally use a no-observed-adverse-effect level (NOAEL) or a benchmark dose as the starting point. For hazards that are considered not to have thresholds for their mode of action, low-dose extrapolation and other modelling approaches may be applied. The default position is that rodents are good models for humans. However, some chemicals cause species-specific toxicity syndromes. Information on quantitative species differences is used to modify the default uncertainty factors applied to extrapolate from experimental animals to humans. A central theme for extrapolation is unravelling the mode of action for the critical effects observed. Food can be considered as an extremely complex and variable chemical mixture. Interactions among low molecular weight chemicals are expected to be rare given that the exposure levels generally are far below their NOAELs. Hazard characterisation of micronutrients must consider that adverse effects may arise from intakes that are too low (deficiency) as well as too high (toxicity). Interactions between different nutrients may complicate such hazard characterisations. The principle of substantial equivalence can be applied to guide the hazard identification and hazard characterisation of macronutrients and whole foods. Macronutrients and whole foods must be evaluated on a case-by-case basis and cannot follow a routine assessment protocol.

Keywords: Hazard characterisation; Chemicals; Mixtures; Micronutrients; Macronutrients

Abbreviations: ADI, acceptable daily intake; AFB_1, aflatoxin B_1; AROI, acceptable range of oral intake; CSTEE, EC Scientific Committee on Toxicology, Ecotoxicology and the Environment; CMC-ENZ, enzymatically depolymerised sodium carboxymethylcellulose; CMC, sodium carboxymethylcellulose; CMG, Common Mechanism Group; Da, Dalton; DEHP, di(2-ethylhexyl)phthalate; DNA, deoxyribonucleic acid; DDT, dichlorodiphenyltrichloroethane; EC, European Commission; EU, European Union; EPA, US Environmental Protection Agency; FAO, United Nations Food and Agriculture Organisation; FDA, US Food and Drug Administration; GABA, γ-aminobutyric acid; GLP, good laboratory practice; GMO, genetically modified organism; GST, glutathione *S*-transferase; HI, hazard index; IARC, International Agency for Research on Cancer; ILSI, International Life Sciences Institute; IPCS, International Programme on Chemical Safety; JECFA, Joint FAO/WHO Expert Committee on Food Additives; LOAEL, lowest-observed-adverse-effect level; MALDI-TOF, time-of-flight mass spectrometry; mRNA, messenger ribonucleic acid; MTD, maximum tolerated dose; NAS, US National Academy of Sciences; NOAEL, no-observed-adverse-effect level; NOEL, no-observed-effect level; NMR, nuclear magnetic resonance; OECD, Organisation for Economic Cooperation and Development; OPP, Office of Pesticide Programs; P450, cytochrome P450; PBTK model, physiologically-based toxicokinetic model; PCB, polychlorinated biphenyl; PCDD, polychlorinated dibenzo-*p*-dioxin; PCDF, polychlorinated dibenzofuran; PE, phytosterol esters; PPAR, peroxisome proliferator activated receptor; RDA, recommended daily allowance; RfD, reference dose; SI, safety index; SL, safety limits; TCDD, 2,3,7,-tetrachlorodibeno-*p*-dioxin; TEF, toxic equivalency factor; TEQ, toxic equivalent; TSH, thyroid stimulating hormone; TTC, threshold of toxicological concern; UL, tolerable upper intake level; WHO, World Health Organization; WOE, weight-of-evidence.

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0278-6915/02/$ - see front matter
PII: S0278-6915(01)00115-6

Contents

1. Introduction

The first stage of a risk assessment, hazard identification, is primarily a question of identifying the effects that are considered as adverse, irrespective of the dose needed or the specific mechanism involved to elicit this effect. The next step, hazard characterisation, is centred on the quantification of these effects, so that the dose–response relationships identified at this stage of the risk assessment can be compared with the potential for exposure (risk characterisation).

The following elements can be identified in hazard characterisation:

- Establishment of the dose–response relationship for critical effects.
- Assessment of external vs internal dose.
- Identification of the most sensitive species and strain.
- Identification of potential species differences (qualitatively and quantitatively).
- Characterisation of the mode of action/the mechanism for the critical effects.

- Extrapolation from high to low dose and from experimental animals to humans.

This approach has been developed for low molecular weight chemicals such as food additives and food contaminants (WHO, 1987). Although the approach is also being applied for micronutrients and nutritional supplements, an additional concern is that deficiency in such components may represent a risk situation. For macronutrients and whole foods, the main principle of the current approach to hazard identification and hazard characterisation is to determine whether the product or process has an equivalent among traditional foods and to establish equivalency by chemical analytical methodologies.

Traditionally, hazard identification and hazard characterisation are based on *data* generated from exposures of experimental animals to individual chemicals. An added dimension in the hazard characterisation of chemicals in food is that exposures are not to single entities, but to a very complex and variable chemical mixture. Thus, in principle, every type of food chemical may exhibit joint similar or joint dissimilar action.

The following report describes the principles of hazard characterisation utilising biologically-based methodology. The first four general sections of this paper describe the main issues in hazard characterisation with focus on low molecular weight chemicals, historically the area with most knowledge and experience. The report starts with an introductory presentation of modes of toxicological action by explaining general mechanisms of toxicity as well as more specific and complex aspects of multiple organ interactions and chemical interactions. The following section describes those causes underlying variability in toxic responses, both within the same species and between different species. Variability in response due to different life-stages or due to different exposure scenarios is also elaborated. In the fourth section, dose–response considerations are discussed, including aspects of adverse vs adaptive responses and threshold vs non-threshold responses. In the fifth section, species differences and interspecies extrapolation are presented, including examples of species-specific toxicity.

The following sections address specific issues in hazard characterisation. Section 6 gives an overview of the current situation for the hazard characterisation of chemical mixtures, explaining mixture terminology and giving an outlook for future developments in chemical mixture assessment. The subsequent two sections describe the special circumstances relating to hazard characterisation of micronutrients and nutritional supplements on one hand and to novel foods, macronutrients and whole foods on the other hand.

2. Fate of toxic chemicals in the body

Studies of the fate of potentially toxic chemicals in the body (toxicokinetics) describe the absorption, distribution, metabolism and excretion of such substances (OECD, 1984). Such data are usually derived from studies using radiolabelled compounds, which are able to follow the absorption, distribution and metabolism of the compound and the excretion of its metabolites in urine and faeces (ADME studies). In recent years, ADME studies have been augmented by chemical-specific analysis of the concentration–time relationships for the chemical, and any active metabolites, in the blood and tissues; such information can be used to define the profile of exposure of the target organ or tissue in relation to the development of toxicity. Toxicokinetic studies on experimental animals are performed to help understand the chemical and biological basis for the toxicological effects observed. It is the ultimate aim of such studies to aid in the assessment of the toxic effects of the test compounds in human beings (WHO, 1986). Comparative toxicokinetic studies on experimental animals and humans often reveal quantitative species differences, especially in metabolism (see section 7.3). Furthermore, toxicokinetic studies may provide data useful for selection of appropriate dose levels for use in other toxicity studies.

2.1. Absorption

Absorption is the process by which an administered substance enters the body. Two elements of the absorption process are of interest, the extent of absorption and the rate of absorption. Absorption of ingested substances through the mucosal cells of the gastrointestinal tract can occur by a variety of mechanisms (IPCS, 1986; Renwick, 1993a; Walsh, 1997). Important characteristics influencing the rate and extent of absorption of a chemical are relative molecular mass, physical state, charge, stability, reactivity (both chemical and metabolic) and solubility. Lipid-soluble chemicals can readily dissolve in the membranes and therefore diffuse across cell walls. In contrast, ionised molecules do not readily enter the lipid–membrane matrix in the ionised form and therefore only the un-inonised form freely diffuses across membranes, except for very low molecular weight substances, which may diffuse through aqueous pores. In general, gastrointestinal permeability correlates with lipophilicity. However, the permeability does not continue to increase for agents with octanol/buffer partition coefficents above 3000 (Walsh, 1997) because such highly lipid-soluble compounds do not form a molecular solution within the gut lumen. The maximal molecular mass taken up by passive diffusion in the gastrointestinal tract is about 3000 Da (Madara, 1995). Specialised transport systems are important to the

absorption of nutrients and endogenous substances (sugars, amino acids, amines, inorganic ions, etc.). A number of organic compounds are subject to active efflux transport, mainly by P-glycoprotein (Fromm, 2000). This reduces their extent of absorption, but is subject to saturation and competition, which can lead to greater absorption than would be predicted. The importance of this process is exemplified by the disposition of avermectin group anthelmintics in P-glycoprotein deficient and wild-type mice (Kwei et al., 1999).

The extent of absorption is the main determinant of the internal dose (which is described in section 6.1). The relationship between the external (applied) dose and the internal dose may be affected by the extent of absorption from the gut lumen, as influenced by lipid solubility and transporters (see above), and also by the extent of any metabolism that occurs in the gut lumen, gut wall or liver, prior to the chemical reaching the systemic circulation (first-pass metabolism). Bioavailability is the parameter that defines the extent of absorption, and reflects both the extent of absorption and of presystemic metabolism. In toxicokinetic terms, the bioavailability of a chemical is defined as the fraction of the administered dose that reaches the systemic circulation as the parent compound, and is usually determined from specially designed toxicokinetic studies (see below). For substances absorbed by passive diffusion, the rate of absorption and the fraction of the dose absorbed from the gut lumen and the rate of absorption are generally independent of the administered dose. However, the fraction of the dose entering the systemic circulation unchanged may show species differences and inter-individual variability due to first-pass metabolism. Dose-dependent absorption kinetics may occur for substances that are absorbed or eliminated by carrier-mediated transport or show dose-dependent effects on gastric emptying rate (although these would usually affect the rate of absorption, but not the extent of absorption), or undergo saturable first-pass metabolic processes.

2.2. Distribution

Distribution is the process by which an absorbed substance and/or its biotransformation products circulate and partition within the body. In order for a toxicant to reach its sites of action, metabolism and excretion, it must traverse one or more cellular membrane barriers. Some barriers are more permeable than others, and some characteristics of the chemicals render them more able to pass through membranes than others. The most important chemical characteristics that affect distribution are lipophilicity, molecular size and shape, and degree of ionisation. Lipophilic, small, non-ionised molecules can diffuse readily across cellular membranes. Very low molecular, hydrophilic substances may diffuse through aqueous pores. Some chemicals are able to utilise active or facilitated carrier-mediated transport mechanisms to enter cells or cross barriers such as the blood–brain barrier or the placenta. A number of tissue parameters affect tissue distribution, such as blood flow rate, the permeability of capillary and cell membranes, and the nature of the extracellular fluid matrix. The tissue distribution of potentially toxic chemicals may be reduced by sequestering processes such as binding to proteins or low molecular weight ligands in plasma, the red cell or in the extracellular space, or by competing elimination (metabolism and excretion) processes (O'Flaherty, 1997). The overall extent of distribution from the general circulation into tissues can be determined from toxicokinetic studies that measure the concentration of the chemical in blood or plasma; the parameter that reflects overall distribution is the apparent volume of distribution. Plasma toxicokinetic studies do not indicate the tissues into which the chemical has partitioned, and this information is usually obtained for animals from studies using the radiolabelled compound, such as autoradiography, or by direct chemical analysis of tissue concentrations. A major advantage of physiologically-based toxicokinetic modelling (PBTK) is its ability to analyse the concentration–time relationships for a chemical in the general circulation and also in tissues, which may show different affinities and perfusion rates.

2.3. Metabolism

Metabolism (biotransformation) is the process by which an administered substance is structurally changed in the body by either enzymatic or non-enzymatic reactions. Many chemicals entering the systemic circulation are lipophilic and weakly ionisable, if at all, and are only poorly excreted because of reabsorption from the renal tubule after filtration in the glomerulus. The biotransformation of xenobiotics generally leads to the formation of more polar, hydrophilic metabolites that may be more readily excreted via the kidneys. The chemical processes underlying metabolism have conceptually been divided into two phases, whereby xenobiotics are oxidised, reduced or hydrolysed in phase I metabolism and conjugated with glucuronic acid, sulfate, acetate, amino acids or glutathione in phase II metabolism. Usually, metabolic conversion leads to a decrease in the toxic potential of an absorbed substance (detoxication). However, some metabolic products of phase I metabolism, and even of phase II metabolism, may be chemically reactive (metabolic activation) and bind irreversibly to tissue molecules leading to various types of toxicities such as tissue necrosis, mutagenicity, carcinogenicity and immunotoxicity (Anders, 1985; Cohen, 1986; Dybing et al., 1989; Hinson et al., 1994; Park et al., 2001). In several instances there are competing metabolic pathways between activation and

detoxication of a foreign chemical. Whether a toxic reaction will be initiated in such situations will depend on the relative balance between these pathways.

The liver is the major site of metabolism of foreign compounds in the body. However, most organs have measurable levels of enzymes that catalyse the reactions involved in biotransformation, including P450 enzymes for oxidation and reduction pathways. For many compounds, organ toxicity arises due to a local activation reaction; organ-specific activation by P450 has been shown to be important in the lung (Boyd, 1980) and the olfactory tissue (Dahl and Haldey, 1991). Conjugation with glutathione, which is usually thought of as a detoxication reaction, is important in the bioactivation of some compounds by the formation of conjugates that are subsequently converted to nephrotoxic metabolites (Anders et al., 1992; Dekant, 1993). It should be noted that most of the metabolising enzymes, including glutathione *S*-transferase (GST) and P450 enzymes, belong to families of related proteins, which can comprise over 50 members in the case of P450. The members of these families are generally under independent genetic regulation, and whilst there is often overlap in specificity, each has a unique substrate profile.

Repeated exposure of chemicals in food may lead to increased synthesis of metabolising enzymes, a process termed enzyme induction. Increased levels of phase I or phase II enzymes may reduce toxicity when this is caused by the parent molecule, but may lead to increased toxicity when this is due to an active metabolite. Some chemicals may inhibit biotransformation enzymes, so that the metabolism of one substance may be impeded by co-exposure to another. Thus, enzyme inhibition may increase toxicity when this is caused by the parent compound, or decrease toxicity that is due to an active metabolite. A number of dietary constituents, including cruciferous vegetables and charcoal-broiled meats, contain components that may act as either enzyme inducers or inhibitors (Conney et al., 1977).

Other factors that might affect metabolism, and thus toxicity, are disease state, age and gender (see section 4.2). A number of phase I and phase II metabolism enzymes display genetic polymorphisms, so that the distribution of xenobiotic metabolising capacity and thus potential toxicity, is not unimodal (Daly et al., 1993). Dependent on the relationship between metabolism and toxicity, some subpopulations of individuals may therefore be uniquely sensitive or resistant to chemical toxicities related to polymorphic enzymes (see section 4.2.1).

2.4. Excretion

Excretion is the process by which an administered substance and/or its metabolites are removed from the body. Ingested compounds that are absorbed will generally be excreted as their metabolites by the kidneys in the urine and/or by the liver in the faeces via the bile. Compounds metabolised to carbon dioxide may be exhaled via the lungs, as can other volatile metabolites. Excretion via maternal milk is important for highly lipophilic contaminants (Gallenberg and Vodicnik, 1989).

Substances present in the blood will be filtered in the kidney glomeruli unless their molecular weights exceed 40–60 kDa. However, if xenobiotics are reversibly bound to proteins in the plasma, they will not be available for filtration. Unless they are actively secreted in the kidney tubules, highly bound compounds will show slower elimination rates than less highly protein bound compounds. Once in the primary urine, depending on lipophilicity and pKa, xenobiotics may be reabsorbed in the tubules and return to the circulation. Substances might also be actively secreted into the proximal tubular lumen through separate transport systems for organic anions and cations. Since some 70–90% of the glomerular filtrate is reabsorbed in the proximal tubule, this could lead to accumulation of xenobiotics and/or their metabolites in the proximal tubular cells resulting to tubular damage (Hook, 1981; Rickert, 1997).

Generally, rats and mice excrete compounds into the bile more extensively than rabbits, dogs or humans, primarily because of species differences in the molecular weight threshold for biliary excretion. In rats, this is approximately 250 Da, whereas in rabbits, dogs and humans it is approximately 400–600 Da (Rickert, 1997). Xenobiotics excreted into the bile as glucuronide conjugates, may re-enter the circulation after being hydrolysed by the gut microflora (enterohepatic circulation).

2.4.1. Kinetic modelling

The term 'toxicokinetics' describes the movement of a chemical around the body. Delivery from the general circulation to the site of toxicity is usually by simple passive diffusion, and therefore the concentrations of the compound in the general circulation reflect the concentrations at the site of toxicity. Definition of the relationship between the external dose and the internal dose removes a large number of variables, such as the effect of the vehicle and route of administration on the systemic body burden, species differences and saturation of elimination, which can influence the overall response. Such information is not obtained from traditional studies using radiolabelled compound, which measure the absorption, distribution, metabolism and excretion of the compound and its metabolites in urine and faeces (ADME studies).

Determination of the toxicokinetics of a compound requires the definition of the concentration–time course for the chemical per se in the body (or in some cases a circulating active metabolite), and therefore is based on measurements of the time course for the changes in the concentration in accessible body fluids, such as blood

and urine. The different processes of absorption, distribution and elimination (metabolism and/or excretion) are reflected in the increases and decreases in the concentrations in such body fluids. The advancement of toxicokinetics has been dependent on the development of analytical methods that are able to measure the low concentrations of the compound present in the plasma, and also the generation of mathematical models able to describe the increases and decreases in concentrations measured. The development of a sensitive analytical method that separates the compound from its metabolites is critical to the generation of valid data. An additional impetus to the study of toxicokinetics was the development of automated analytical facilities capable of processing the large number of samples that are generated as a result of the multiple sequential blood sampling, which is a feature of toxicokinetic studies.

Historically, three different approaches have been used to analyse the plasma concentration–time curves in order to define the basic kinetic processes (Renwick, 1993a).

2.5. Multi-compartmental analysis

Compartmental analysis involves the fitting of simultaneous exponential equations to the plasma concentration–time curve data, without an a priori determination of the relationship between the rate constant and any physiological or metabolic processes. The entry of the chemical into the system is usually described by a simple rate constant which may be either first-order (proportional to the concentration) or zero-order (constant and independent of the concentration). In most models the chemical is assumed to enter a central compartment (equivalent to the blood plus rapidly-equilibrating tissues), prior to distributing into a second compartment (usually representing slowly equilibrating, poorly-perfused tissues). Elimination is normally from the central compartment and is usually assumed to be a first-order reaction. An advantage of compartmental analysis is that it allows the plasma concentration–time curve data to be extrapolated, so that the concentration present at any time after dosing can be calculated from the model fitted to the experimental data. Also parameters such as the area under the plasma concentration–time curve (AUC) and the clearance (CL) and the apparent volume of distribution (V) can be used to predict steady-state body burdens. A disadvantage of compartmental analysis is that it is not readily related numerically to physiological or metabolic processes that occur within the body.

2.6. Non-compartmental analysis

In non-compartmental analysis, the toxicokinetics of the compound are defined without the necessity of fitting the data to a particular multi-compartment model. The parameters are derived by a combination of direct measurement of the AUC, and simple regression analysis to estimate the terminal half-life (which is used to extrapolate the AUC to infinity). The output of such models includes the most important toxicokinetic parameters, such as the bioavilability (F; which is a measure of the fraction of the administered dose that reaches the general circulation as the parent compound), the plasma clearance (CL; which reflects the activity of all processes contributing to the elimination of the chemical from the general circulation), the apparent volume of distribution (V; which reflects the extent to which the chemical partitions reversibly from the general circulation into body tissues) and the elimination half-life ($t_{1/2}$: which is proportional to the CL but inversely proportional to V, and determines the time to reach steady state during repeated dosage). These parameters can be used to extrapolate from a single dose to steady state (chronic) exposure.

2.7. Physiologically-based toxicokinetic models (PBTK models)

PBTK models use a series of equations to describe each of the physiological processes involved in the absorption, distribution and elimination of the compound. The absorption rate is based on the physicochemical properties of the compound and the site of administration; distribution is described by a series of equations relating to the blood flow (Q) to the major body organs, and the partition coefficient for the uptake of the chemical by the tissue (usually measured from in vitro studies); elimination is described by appropriate rate constants, which can include Michaelis–Menton constants. The development of such as model requires a detailed understanding of the biological fate of a chemical in the body, for example, the sites of tissue distribution and the enzymes involved in its elimination. Thus a large amount of chemical-specific information is necessary to develop an appropriate physiologically-based model prior to comparing the output of the model with actual measured plasma concentration–time curve data. Because of the complexity of the numerous tissue compartments with their blood flow and partition coefficients, it is usual to develop simplified models. These simplified models combine or pool tissues that share similar perfusion rates and partition coefficients, and therefore combine aspects of both full PBTK models and the simpler multi-compartment models. A major advantage of PBTK models is that in vitro data on enzyme kinetics from both animal and human tissues can be incorporated into the model. This means that a model can be developed using in vitro data for animal tissues, validated against in vivo animal data, and then extrapolated to humans using in vitro human data, without the need for in vivo exposure to humans.

2.8. The incorporation of toxicokinetic data into hazard characterisation

Toxicokinetic data provide quantitative information on the magnitude of species differences and inter-individual variability in the relationship between the external dose and the internal dose, both after a single dose and also during repeat-dose toxicity studies. In addition, the influences of variables, such as the vehicle and route of administration, can be quantitated. Such quantitative data have the potential to make major improvements to hazard characterisation by the removal of many of the variables and uncertainties associated with the use of in vitro and in vivo animal data (Renwick and Lazarus, 1998). Currently, toxicokinetic data are a routine part of the database supplied for the assessment of human and veterinary medicines. However, to date the amount of such information provided for low molecular weight chemicals present in the food, such as pesticide residues and some food additives, is much more limited. This is often restricted to information on fraction of absorbed dose (but not bioavailability of parent compound) and elimination (half-life and/or clearance) of total radiolabel, occasionally half-life of parent compound. It is very unusual to obtain any information on enzyme specificity or interindividual variability in any of the kinetic or metabolic parameters. However, increasing awareness of the importance of kinetics and metabolism as determinants of interspecies and interindividual differences in toxicity makes it likely that, in the future, a more comprehensive description of the kinetics and metabolism of such compounds will be required.

Toxicokinetic data can be incorporated into the mathematical approaches used for risk assessment, such as the application of uncertainty factors or dose–response extrapolation. For example, default uncertainty factors can be replaced by chemical-specific adjustment factors, or dose–response relationships can be extrapolated using estimates of the internal dose (or even the estimated human target organ dose), instead of the external dose. These approaches are discussed in greater detail in Edler et al. (2002).

3. Modes of toxicological action

3.1. General mechanisms of toxicity

Most toxic agents produce their effects through the disruption of cellular and molecular processes responsible for homeostasis. Initial reactions may be impairment of household functions such as metabolic rates, cell growth or gene transcription. Further disruption of homeostatic processes can result in an array of effects that include alterations in basic cellular functions that typify the function of a particular target organ. In addition, other toxicant-induced effects can include altered cellular repair mechanisms, altered cell proliferation and general cytotoxicity. The effects of toxic agents on living systems are the result of multifaceted biological interactions with biochemical, cellular and molecular processes. Although we tend to describe mechanisms in a singular fashion, toxicity is often the consequence of concurrent or sequential aberrations in more than one biochemical, cellular or molecular pathway (see also Eisenbrand et al., 2002). Often the term "mode" of action is used to describe the key events and processes involved in toxicity. This is contrasted with "mechanism" of action, which implies a more detailed, molecular description of events than is meant by mode of action (EPA, 1999). Generally, for hazard characterisation purposes, it is not necessary to know precisely the mechanism of action, it will suffice to have an understanding of the mode of action.

3.1.1. Dysregulation of cellular homeostasis

The concept of altered cellular homeostasis and its relation to cell injury has been examined in depth by many investigators. This concept has now come to be accepted as one of the mechanisms by which diverse toxicants share a common path towards cell injury and death. A number of cellular changes can be induced by toxicants, either by altering the intracellular concentration of cellular constituents such as electrolytes or by altering the concentration of endogenously produced substances such as hormones in the immediate surrounding of a cell. For example, dysregulation of intracellular calcium concentration leads in a first step to cytoskeletal changes and bleb formation, nuclear chromatin clumping, and mitochondrial condensation. At a later stage, following a rise in intranuclear calcium concentration, DNA fragmentation and subsequent progression into programmed cell death (apoptotic) pathways can occur (Corcoran and Ray, 1992; Trump and Berezesky, 1995).

3.1.2. Receptor-mediated mechanisms

The toxic effects of many compounds can be explained via receptor-mediated actions at the level of the plasma membrane or the cytosol. The neurotoxic effects of cyclodiene insecticides such as dieldrin and heptachlor are thought to produce their effects through antagonistic interactions with membrane-bound γ-aminobutyric acid (GABA) receptors, specifically $GABA_A$-receptors (Eldefrawi and Eldefrawi, 1987). These inhibitory receptors modulate chloride ion flux through a voltage-dependent chloride channel. The antagonistic interaction of cyclodiene pesticides with the $GABA_A$-mediated chloride channel can result in dysinhibition and subsequent neurotoxic effects such as excitation or convulsions. Biological toxins, such as tetrodotoxin from the puffer fish, or saxitoxin from dinoflagellates, impair

sodium channels in excitable cells, thereby blocking the action potential (Ritchie, 1980). Dichlorodiphenyltrichloroethane (DDT), a chlorinated hydrocarbon insecticide, exerts its toxic effects by slowing the closing of the sodium channel and thereby altering the repolarization process in excitable membranes (Matsamura, 1985).

Association with the cytosolic arylhydrocarbon (Ah) receptor is the initial step by which halogenated aromatic hydrocarbons such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and its analogues exert their effects on the cell (Okey et al., 1994). The Ah receptor exists as part of a soluble cytosolic protein complex that binds compounds such as TCDD. Binding of the ligand to the receptor complex is followed by a series of steps that results in nuclear translocation of the ligand–receptor complex. Subsequent binding of the nuclear form of the Ah receptor–ligand complex to DNA enhancer sequences results in transcriptional activation and/or repression altering the synthesis of a number of cellular proteins. These proteins include certain drug-metabolising enzymes (both P450-mediated and non-P450-mediated) and growth regulatory proteins such as epidermal growth factor receptor, transforming growth factor α and interleukin 1β.

Steroid hormones (e.g. estradiol and testosterone), synthesised and secreted by the gonads, control many fundamental events, such as the development and differentiation of the female and male reproductive system. The primary step in their mechanism of action is binding to specific intracellular receptors. A variety of compounds (industrial chemicals, environmental pollutants, plant oestrogens) can also interact with these receptors, and hence have the potential to interfere with reproductive development by mimicking or inhibiting the effects of steroid hormones, a process termed endocrine disruption. Via binding of these chemicals to the steroid hormone receptors they can trigger responses that are qualitatively similar but by far less potent than that induced by the hormone itself. For example, various pesticides (chlordecone, DDT) and plant oestrogens (lignans, flavonoids, e.g. daidzein, genistein) as well as mycotoxins (zearalenone) can bind to the oestrogen receptor but show a much lower oestrogenic activity than the endogenously synthesised oestradiol. Other compounds may strongly bind to a hormone receptor and thereby prevent the interaction of the endogenous ligand with this receptor. This is known to be the case of the major and persistent DDT metabolite 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (DDE), as well as that of two metabolites of the fungicide vinclozolin, which act as anti-androgens by competing effectively with endogenous androgen for androgen receptor binding.

3.1.3. Cell membrane-mediated effects

Cell membranes are common targets for toxicant-induced injury. As the primary barrier between the cell and its external environment, toxicants must cross the cell membrane to gain entry into the cell and toxicants often interact with specific components of the cell membrane. Targets other than specific membrane-bound receptors can be affected by toxicants. Some compounds, such as hypnotic agents and organic solvents, produce central nervous system depressant effects via non-specific alterations in membrane fluidity (Hobbs et al., 1996).

3.1.4. Alterations in cell energetics

Cellular energy production and subsequent energy utilisation are vital to the survival of all cells. Some cell types within the brain, heart and kidney are particularly susceptible to the effects of toxic agents when their capacity to produce and utilise energy substrates is diminished. Any compound that either directly or indirectly affects these mechanisms has the potential to produce adverse effects. For example, malachite green, an *N*-methylated diaminotriphenylmethane dye, has been shown to promote dissipation of the mitochondrial membrane potential, which results in enhanced mitochondrial permeability and swelling followed by respiratory inhibition (Kowaltowski et al., 1999).

3.1.5. Binding to critical cellular macromolecules

Covalent binding of toxicants to critical cellular macromolecules is a well documented and accepted mechanism of toxicant-induced injury. The role of reactive metabolite binding to cellular constituents and subsequent tissue necrosis is well characterised and is known to be involved in target organ specificity of injury (Brodie et al., 1971). Toxicant binding usually occurs with structural proteins, critical enzymes, lipids and/or nucleic acids. The binding interaction is usually between an electrophilic reactive intermediate and a nucleophilic thiol, amino or hydroxy group. Covalent binding is thought to be an irreversible process when the binding overwhelms the capacity of the cellular repair mechanisms. Interaction of reactive electrophiles with nucleophilic sites in DNA can result in genotoxicity (Miller and Miller, 1985). Aflatoxins represent a group of closely related mycotoxins produced by the common fungal molds *Aspergillus flavus* and *A. parasiticus*, aflatoxin B_1 (AFB_1) being the most toxic and carcinogenic among them. AFB_1 is inactive per se but can be metabolised to AFB_1-8,9-epoxide, which in turn can bind to proteins (thereby eliciting cytotoxicity) and to DNA (thereby leading to genotoxicity and in the long term to liver cancer) (Eaton and Gallagher, 1994).

3.1.6. Oxidative stress

Oxidative stress is defined as "a disturbance in the prooxidant–antioxidant balance in favour of the former, leading to a potential damage" (Sies, 1997). This definition leaves open the possibilities of the disturbance resulting from increases in oxidant production or

decreases in tissue reductive capacity, or a combination of these processes. Oxidative damage is mediated by free radicals. The ubiquitous nature of oxygen, and the rapid reaction of most organic radicals with this molecule, results in oxygen-centred radicals being the most common type found in biological systems. However, other atoms found in organic molecules such as carbon, nitrogen, sulphur and phosphorus can and do exist as free radicals. Free radicals can react with proteins, lipids and DNA. Modifications to these molecules can, in turn, affect various signal transduction systems as well as defence and repair enzymes. The final result ranges from adaptation to a return to homeostasis to permanent injury and even death.

In this respect, ferrous and ferric iron, which are known to catalyse the formation of $^{\bullet}OH$ radicals (via the Fenton and Haber–Weiss reactions, respectively) induce lipid peroxidation of isolated organelles (mitochondria, lysosomes) and hepatocytes in vitro (Goering and Klaassen, 1997). Under conditions of iron overload, in vivo lipid peroxidation has also been shown to occur and to be responsible for functional deficits such as perturbations of mitochondrial oxidative metabolism and increased lysosomal fragility (Goering and Klaassen, 1997). Furthermore, chronic iron overload has been associated with development of hepatocellular carcinomas in hemochromatosis patients due to genetic disease. This effect could be related to the ability of iron to initiate production of reactive oxygen intermediates and/or iron-catalysed DNA damage (Goering and Klaassen, 1997).

3.1.7. DNA repair inhibition

Several metals including nickel, cadmium, cobalt and arsenic have been shown to be carcinogenic to humans and/or experimental animals (IARC, 1990, 1991, 1993). Recent studies suggest that DNA repair systems are very sensitive to nickel (II), cadmium (II), cobalt (II) and arsenic (III), thereby leading to a less efficient removal of endogenously formed DNA lesions as well as of DNA lesions induced by environmental agents and to an increased risk of tumour formation (Hartwig, 1998). However, the underlying mechanisms are different for the various above-mentioned metals. Nickel (II) and cadmium (II) disturb the first step in the nucleotide excision repair process, namely the recognition of DNA damage (Hartmann and Hartwig, 1998). Arsenic (III) inhibits the incision step at low and the ligation step at higher concentrations (Hartwig et al., 1997), whereas cobalt (II) inhibits the incision and the polymerisation steps (Kasten et al., 1997).

3.2. Multiple secondary organ interactions

Compounds may exert in a first step a toxic effect in one organ and subsequently affect the function of further organs. This is, for example, the case of the polyols sorbitol or xylitol (Roe, 1984; Roe and Bär, 1985). If rats are fed high concentrations of sorbitol or xylitol, enlargement of the cecum and increased absorption of calcium from the gut are observed, subsequently leading to increased urinary excretion of calcium, pelvic and corticomedullary nephrocalcinosis, acute tubular nephropathy and urinary calculus formation. Also hyperplasia and neoplasia of the adrenal medulla are observed. The adrenal medullary proliferative disease in rats is usually seen concomitantly with multiple endocrine neoplasia, with the pituitary gland, the pancreatic islets and the thyroid C-cells being most commonly affected.

3.3. Multiple chemical interactions

Whenever a living organism comes into contact with more than one toxic substance at a time in sufficiently high doses, one has to consider the possibility that the compounds interact and thereby modify the toxicity of one or both substances. Chemical interactions are known to occur by a number of mechanisms such as alterations in absorption, protein binding, biotransformation or excretion of the interacting toxicants, and will be covered in section 6.

3.4. Concluding remarks

In the present section, general modes of action underlying the toxicity of a variety of compounds have been presented. The elucidation of the precise mechanism per se only represents one milestone in the entire risk assessment process, but when put into context with the results of acute, subchronic and chronic toxicity testing as well as with the reproductive toxicity, immunotoxicity, genotoxicity and carcinogenicity data sets (see Barlow et al., 2002) enables the toxicologist to obtain a very precise profile of the compound tested. This "integrative" approach, in which results derived from mechanistic studies and the various toxicity endpoints mentioned above are taken into account to assess the potential hazard of a compound present in food, will help in the future to identify more readily the causes underlying variability in toxic responses (an issue covered in section 3), and will allow a better extrapolation of the toxicity data obtained in experimental animals to humans, thereby leading to an optimisation of the risk assessment process.

This section gave a general description of possible modes of action of chemicals on the way to exert adverse effects in an organism. These are the early events, and it has to be kept in mind that toxicity is often the consequence of concurrent or sequential events as described above. Detailed knowledge on the mechanism of toxicity will allow identification of the

sequence of events as well as the overall mode of action of a compound. Overall, this will help to identify the critical step on which further considerations for hazard characterisation can be based on in relation to considerations of variability and dose response, before species extrapolations can be performed.

4. Causes underlying variability in toxic responses

4.1. Interindividual variability in response to differences of toxicokinetic and toxicodynamic nature between species

4.1.1. General physiological differences

Many toxicants are able to injure one organism without harming others, this selectivity being in part explained by the fact that the compound may react fairly specifically with the one organism due to physiological or biochemical characteristics which are absent or do not play an important role in other organisms. Selective toxicity due to differences in physiology is exemplified by comparison of plants and animals. Plants differ from animals in many ways; for instance, the absence of a nervous system, an efficient circulatory system and muscles and the presence of a photosynthetic mechanism and rigid cell walls. The animal toxicity of many insecticides results from their effects on the nervous system; plants therefore are relatively insensitive. On the other hand, animals are relatively insensitive to most herbicides. The fact that bacteria contain cell walls and humans do not has been used in developing selective toxic chemotherapeutic agents, such as penicillin and cephalosporins, that kill the bacteria but are relatively non-toxic to mammalian cells.

Selective toxicity can also be the result of differences in basic biochemical pathways present in two organisms. For example, bacteria do not absorb folic acid but synthesise it from *p*-aminobenzoic acid, glutamic acid and pteridine, while mammals cannot synthesise folic acid and have to absorb it from their diet. Sulfonamides are toxic to bacteria because they resemble *p*-aminobenzoic acid in charge and dimensions, thereby antagonising the incorporation of *p*-aminobenzoic acid into the folic acid molecule, a reaction that humans do not carry out.

4.1.2. Interspecies variability

Variability in the response of different animal species to a certain toxicant can be due to differences in compound uptake, distribution, accumulation, metabolism, excretion or target sensitivity, differences in metabolism representing the most frequent explanation for the observed qualitative and quantitative differences in toxic effects among animal species. For example, 2-naphthylamine is first hydroxylated to the corresponding *N*-hydroxylamine via a cytochrome P450 1A2-catalysed reaction. In a second step the *N*-hydroxylamine is converted by cytosolic *N*-acetyltransferase to the highly unstable *N*-acetyloxynaphthylamine, which degrades to the highly reactive arylnitrenium ion. Rapid acetylators within the human population catalyse *N*-acetylation very efficiently, whereas rats are known to be slow acetylators. Dogs are deficient in cytosolic *N*-acetyltransferase; however, a microsomal *N*,*O*-acyltransferase as well as bacterial deacetylases are able to carry out the above-mentioned activation in this animal species. Taken together, these observations very well explain the fact that 2-naphthylamine is a much more potent carcinogen in humans and dogs than in rats (see also section 5).

There are many physiological and anatomical similarities between laboratory animals and humans which justify the use of animals in toxicological evaluations. However, one cannot ignore the degree to which qualitative and quantitative differences affect interspecies extrapolation (Oser, 1981). Some of these differences are mentioned in Table 1, in this example, characteristics of the laboratory rat, which do not apply to humans. See also section 5 for a detailed discussion on species differences and interspecies extrapolation.

Table 1
Interspecies differences: characteristics of the laboratory rat, which do not apply to humans

General differences	*Physiological differences*	*Behavioural differences*
Small size	Multiparous	Nocturnal
Prolific breeder	No emetic reflex	Coprophagy
Brief gestation/lactation		Cannibalism
Short lifespan	*Biochemical differences*	
Dry diet acceptable	α2μ-Globulin formation in males	*Genetic variability*
		Strain variabilities in spontaneous tumours and intercurrent infections
Anatomic differences	*Nutritional differences*	
Lack of gall bladder	Different mineral and vitamin requirement	
Yolk-sac placenta	Independent of dietary ascorbic acid	*Pattern of living conditions*
Multiple mammae		Under controlled light/temperature/humidity
Forestomach		
Fur-bearing		

4.2. Variability in response within population

4.2.1. Genetic

Variabilities in the levels of expression of enzyme activities associated with the activation and/or detoxification of foreign compounds profoundly affect the toxic responses of individuals towards these substances. These variabilities can be due to genetic differences among individuals. Heritable genetic differences that occur at a level of $\geqslant 1\%$ in a population are defined as genetic polymorphisms. Genetic polymorphisms can be caused by mutations in the coding regions of structural genes, mutations in the regions where transcription factors bind, mutations in the coding regions of genes that act as *trans* regulatory elements, introduction of premature stop codons or even deletions of whole genes (or large parts of genes), base-pair mutations giving rise to abnormal mRNA splicing and gene duplication. For example, *N*-acetylating enzymes biotransform a wide variety of drugs and other chemicals with primary aromatic amine or hydrazine groups. Because some of these substances are known to be carcinogenic, particularly for bladder cancer, *N*-acetyltransferase polymorphisms have been examined in detail. Significantly increased frequencies of "slow" acetylators among bladder cancer patients compared with controls have been reported in many populations (Lower et al., 1979; Cartwright et al., 1982; Hanke and Krajewski, 1990; Hayes et al., 1993; Raunio et al., 1995). In contrast, an excess of "fast" acetylators have been found among patients with colon cancer and colonic polyps (Lang et al., 1986; Ilett et al., 1987; Wohlleb et al., 1990; Lang et al., 1994).

The condition of favism takes the form of haemolytic anaemia, haematuria and jaundice following consumption of fava beans (broad beans; *Vicia fava*) by some individuals. The mechanism is as follows: the bean contains two heterocyclic compounds, vicine and covicine, which oxidise glutathione, notably in the erythrocyte. If this occurs to the extent that the redox status is significantly affected, oxidation of erythrocyte membrane lipids and subsequent haemolysis occur. In normal individuals, the oxidised glutathione is converted back to the reduced form by glutathione reductase, which in turn depends on reducing equivalents derived from glucose-6-phosphate dehydrogenase. A sensitive subpopulation is glucose-6-phosphate dehydrogenase-deficient and as a result are unable to regenerate reduced glutathione at the required rate under stress by vicine/covicine. Therefore, episodes of favism have arisen in such individuals, with occasional fatalities. The condition of glucose-6-phosphate dehydrogenase deficiency is particularly common among people of Mediterranean and Asian origin.

4.2.2. Gender

Pronounced sex differences in the P450-mediated metabolism of some xenobiotics have been observed in rodents (Gustafsson et al., 1983). These sex differences in xenobiotic metabolism are controlled by the hypothalamus and pituitary gland (Gustafsson et al., 1983). Correlating with these observations is the fact that a number of substances have been shown to be carcinogenic in only one of the two sexes tested in mice and rats. Sex differences associated with metabolism have also been observed in other species, including humans (Wrighton and Stevens, 1992), but have not been as well studied. In general, pronounced consistent gender differences in xenobiotic metabolism in humans have not been reported.

A non-endocrine-dependent example of sex differences in carcinogenicity is *d*-limonene, a naturally occurring monoterpene hydrocarbon found in a variety of citrus fruits and used widely as a flavour, fragrance and industrial solvent that leads to $\alpha_{2\mu}$-globulin nephropathy exclusively in male rats (Lehman-McKeeman, 1997). Acute exposure to *d*-limonene results in the accumulation of $\alpha_{2\mu}$-globulin in renal proximal tubule cells leading to protein overload progressing to renal cell injury, compensatory cell proliferation and ultimately a low but significant incidence of renal tubular tumours. The species and gender specificity of this nephropathy is determined by the fact that $\alpha_{2\mu}$-globulin is only synthesised by adult male rats.

4.2.3. Nutritional status

The bioavailability of a number of compounds can strongly be influenced by the nutritional status. For example, gastrointestinal absorption of cadmium is enhanced by a dietary iron deficiency; in this context, women with low serum ferritin levels have been shown to have twice the normal absorption of cadmium (Flanagan et al., 1978). Phytic acid, a major phosphorus storage compound of most seeds and cereal grains, contributes about 1–7% of their dry weight and has the ability to chelate multivalent metal ions, especially zinc, calcium and iron. The binding can result in very insoluble salts that are poorly absorbed from the gastrointestinal tract, thereby strongly reducing the bioavailability of the above-mentioned minerals (Zhou and Erdman, 1995).

Mineral deficiencies (calcium, copper, iron, magnesium and zinc) decrease both cytochrome P450-catalysed oxidation and reduction reactions. Decreases in basal cytochrome P450 concentrations can in part account for the lower biotransformation activity. A restoration to normal dietary mineral intake returns the enzyme activities to physiological levels.

Vitamin deficiencies (C, E and B complex) reduce the rates of xenobiotic biotransformation. These vitamins are directly or indirectly involved in the regulation of the cytochrome P450 system. In addition, their deficiencies can alter the energy and redox state of the cells, thus hindering the production of the high-energy factors

required for phase II biotransformation. Reintroduction of the vitamins to the diet will result in a return to basal enzyme activities. On the other hand, substances present in food may interfere with the endogenous activity of certain vitamins. For example, a reduction of vitamin K-dependent blood clotting activity leading to hemorrhagic death has been observed in rats fed antioxidants such as butylated hydroxytoluene and these effects can be prevented by supplementation of food with vitamin K (Cottrell et al., 1994).

Low-protein diets have been found to increase markedly the toxicity of a number of xenobiotics that are active as the parent compound, but to reduce the toxicity of those that require biotransformation to express their toxicity. For example, the lethality and severity of hepatotoxicity produced by dimethylnitrosamine are markedly reduced in rats maintained on a low-protein diet. Correlated with these decreases in toxicity is a reduction in the cytochrome P450-mediated *N*-demethylation of dimethylnitrosamine, the initial step in its conversion to an alkylating agent. A limited number of studies suggest that the effects of protein malnutrition on chemical toxicity in humans are also related to changes in the activities of cytochrome P450 isoforms.

Protein deficiency may also lead to an enhanced toxicity of a number of compounds. Methylation has been considered to be the primary mechanism of inorganic arsenic biotransformation and detoxification in most animals, since the methylated metabolites of inorganic arsenic are less acutely toxic, more quickly excreted and demonstrate less tissue reactivity. Protein deficiency results in decreased methylation and enhanced toxicity of inorganic arsenic (Wildfang et al., 2000). Numerous disease states such as Kunzo and tropical ataxic neuropathy have been proposed to be due to chronic exposure to cyanide via cyanogenic foodstuffs (cassava). These disease states are more frequent and more severe if the intake of dietary protein is low. Under this condition, the plasma concentration of cysteine and the amount of 3-mercaptopyruvate, which is derived from cysteine and which is needed to bind and detoxify cyanide, are decreased (Isom and Baskin, 1997).

Dietary (caloric) restriction on the one hand enhances longevity in experimental animals (Dreosti, 1998; Lipman et al., 1998), and on the other hand reduces tumour incidence in several laboratory animal species, this being true for a variety of tumour types and for both spontaneous tumours and tumours caused by diverse types of tumour-inducing agents (Lipman et al., 1998; Kari et al., 1999). In contrast, the relationship between overfeeding, excessive body weight and poor survival are now well established and have been demonstrated in every rodent strain and stock examined (Keenan et al., 1997).

Dietary lipids are important in determining the activity of biotransformation enzymes, particularly those enzymes that are membrane bound. In rats, diets high in polyunsaturated fats decrease the concentration of hepatic cytochrome P450. This reduction results from the increased susceptibility of unsaturated fatty acids to undergo peroxidation. Thus, the microsomal membranes degrade with a concomitant loss of cytochrome P450. The nature of the fatty acids present in the membrane may also affect its fluidity and thus toxicity of membrane-perturbing agents.

Natural compounds present in vegetables and fruits are known to induce various phase II drug metabolizing enzymes, thereby leading to the inactivation of toxic compounds being taken up with food. 1,2-Dithiol-3-thiones, which are natural constitutents of cruciferous plants such as cabbage and Brussels sprouts and potent inducers of GSTs, protect rats being treated concomitantly with the liver carcinogen aflatoxin B_1 by trapping the ultimate carcinogenic metabolite aflatoxin B_1-8,9-epoxide with glutathione (Kensler et al., 1987, 2000; Manson et al., 1997).

An alternative mechanism by which natural compounds might protect an organism from toxic compounds present in food is the selective stimulation of growth of one or a limited number of bacterial strains in the colon. For instance, inulin, a term applied to a group of fructose polymers [so-called β(2-1) fructans] found widely distributed in nature as plant storage carbohydrates, is resistant to digestion by pancreatic and intestinal hydrolases. Once in the colon, inulin is completely fermented, thereby leading on the one hand to a preferential growth of *Bifidobacteria* and *Lactobacilli*, and on the other hand to an enhanced butyrate generation (Kruse et al., 1999; Videla et al., 2001). *Bifidobacteria* and *Lactobacilli* have lower azoreductase, nitroreductase, β-glucuronidase, β-glucosidase and 7α-hydroxylase activities than a number of *Bacteroides*, *Clostridium* and *Enterobacterium* strains (Rowland, 1991). As the above-mentioned enzymes are involved in the activation of various toxic food contaminants, the prevalence of *Bifidobacteria* and *Lactobacilli* could result in a reduced colon cancer risk, which in fact has been shown to be true in various chemically-induced colon carcinogenesis models (Pool-Zobel et al., 1996; Singh et al., 1997; Rowland et al., 1998). Furthermore, butyrate has been shown to induce apoptosis in colon tumour cell lines (Hague et al., 1995; Hague and Paraskeva, 1995), and very recently experimental evidence has been presented that inulin does stimulate apoptosis in 1,2-dimethylhydrazine-treated rats (Hughes and Rowland, 2001).

4.2.4. Health status

Liver disease obviously has a major influence on xenobiotic metabolism (Howden et al., 1989). There are three primary factors involved in the alteration of xenobiotic metabolism by liver disease. Changes in liver

blood flow affect the delivery of the xenobiotic to the site of metabolism. A reduction in metabolic capacity is likely to occur in liver disease due to a diminished number of viable hepatocytes. Additionally, albumin production is frequently diminished in patients with liver disease, potentially resulting in a higher concentration of unbound drug. This can lead to higher tissue concentrations of the drug and enhanced toxicity (Howden et al., 1989). Other disease states such as diabetes and hypertension can also lead to changes in xenobiotic metabolism (Schenkman et al., 1989). Stress has also been shown to produce changes in xenobiotic metabolism and immunotoxicity (Vogel, 1993). In many human populations, in which primary liver cancer occurs at high incidence, hepatitis B virus infection and high levels of aflatoxin ingestion exist concurrently. Available evidence indicates that risk for primary liver cancer developing in these populations may be strongly amplified through synergistic effects of aflatoxin ingestion and hepatitis B virus infection, this synergism being most probably due to the increased rate of cell proliferation resulting from hepatitis (Ross et al., 1992).

Glomerular filtration and tubular secretion of xenobiotics usually fall in concert with renal impairment in kidney disease leading to reduced clearance of many chemicals (Ritter et al., 1993). The decline in excretion of chemicals is directly related to the glomerular filtration rate. The metabolism of several xenobiotics is also reduced in the event of renal failure, but is probably of little significance. Uraemia consequent to kidney disease might be associated with an increase in the permeability of the blood–brain barrier (Hawkins, 1986).

4.2.5. Xenobiotic co-exposures

Whenever an organism is exposed to more than one compound at a time, one has to consider the possibility that these substances show joint actions (Groten et al., 2000). The joint action can result in: (1) Dose addition: each of the chemicals in the mixture acts in the same way, by the same mechanism(s) and only differ in their potencies. Simple joint action allows the additive effect to be described mathematically, using the summation of the doses of the individual compounds in the mixture, after adjustment for differences in potencies. (2) Response/effect addition: the modes of action and possibly the nature and site of action differ among the chemicals in the mixture; these exert their individual effects without modifying the effects of the other constituents in the mixture. Mathematically, effect addition is the summation of the responses to each compound in the mixture. (3) Stronger or weaker effect than expected on the basis of either dose or response additivity may occur. See also section 6 for a review on hazard assessment of chemical mixtures in food.

4.2.6. Variability in sequential events

The final response that is observed in an experimental animal dosed with a chemical depends on the interaction of a complex series of events, especially with effects that occur only after prolonged exposure such as tumour formation. The series of events can influence the shape of the dose–response curve and the variability of response. In general, the shorter the chain of events the steeper the dose–response curve and the lower the variability. In fact for quantal effects such as death in acute studies or the induction of tumours in chronic studies, the slope of the dose response actually describes the experimental variability through heterogeneity of the experimental conditions. If there were no variability, the slope of the dose–response curve would be vertical as all of the animals would change from being non-responders to responders at the same dose.

The sources of variability arise from the series of events that lead to the toxic effect. This can be illustrated with an example of a compound that causes tumours in the kidney. The following chain of events may occur (Anders et al., 1992; Dekant, 1993; Green et al., 1997):

1. The compound is taken in by ingestion and then a proportion of it is absorbed.
2. After being absorbed a proportion of the absorbed dose is conjugated to glutathione in the intestinal wall.
3. The conjugate then enters the blood stream and a proportion is filtered in the kidney.
4. The conjugate passes through the tubule wall and a proportion is activated by β-lyase to produce an active metabolite.
5. A proportion of the active metabolite is not inactivated and reacts with the kidney tubule cells.
6. A proportion of these cells are damaged by the active metabolite.
7. A proportion of these cells cannot repair the damage and die.
8. A proportion of the new cells, which are produced to replace the dead cells, carry a mutation (spontaneous, induced by the active metabolite or another compound) which affects their growth control mechanism.
9. A proportion of these cells survive DNA repair mechanisms and proliferate.
10. A proportion of the proliferating cells become cancerous and a tumour results.

At each of the 10 steps in this example there is the possibility for the step to be below the level at which the next step would continue, and therefore the animal will not show the subsequent effect. In addition there are 10 steps in which there can be variability and the total

variability might be larger than the sum of each of the individual variabilities.

4.3. Variability in response due to different life stages

During pregnancy, physiological changes occur in virtually every maternal organ system as a consequence of, and in order to support, the rapid growth of the foetus and reproductive tissues, and these changes may profoundly influence the fate of toxicants. Early in pregnancy, maternal gastrointestinal motility becomes depressed and as a consequence may result in enhanced absorption of hydrophilic compounds that are generally poorly absorbed. During pregnancy the volume of distribution is generally increased due to extensive increases in various body tissue and fluid volumes. Thus, initial concentrations of hydrophilic drugs will tend to be lower during late compared to early pregnancy. The higher fat content increases the potential for greater body burden of lipophilic compounds. Owing mainly to the increase in plasma volume, the plasma concentration of protein, mainly albumin, decreases to low levels during the third trimester. Therefore, decreased plasma albumin results in more unbound toxicant being available for placental transfer and, at least during the early part of lactation, excretion into milk. During pregnancy the pH of the maternal plasma remains fairly constant, but the pH of the embryo/foetal compartment changes from a basic one, relative to maternal plasma, during early organogenesis to a more acidic environment during late organogenesis and foetal development. Therefore, placental transfer and potential accumulation of weakly acidic chemicals is favoured during embryogenesis, while weakly basic chemicals are more likely to be transferred during late gestation. Renal blood flow, as well as the glomerular filtration rate, increase during pregnancy, these profound changes leading to an enhanced renal toxicant clearance such that plasma concentrations fall more rapidly as pregnancy progresses. In this respect one exception is caffeine: its clearance decreases during pregnancy (Knutti et al., 1982).

Following parturition, increases in mammary blood flow and milk production strongly influence the amount of toxicant being transferred into milk. The amount and rate of transfer of a toxicant into milk depends on its pK_a, lipophilicity, molecular weight and on the degree of protein binding and pH gradient between plasma and milk. Of particular concern for neonatal exposure are lipid-soluble toxicants that had previously accumulated in fat stores during pregnancy, such as dioxins. As the body fat content gradually declines to non-pregnancy levels following parturition, the concentration of lipophilic toxicants in the rest of the body increases, causing enhanced potential for lactational transfer of toxicants at harmful levels.

While the liver remains the major site of drug metabolism throughout pregnancy, the developing foetoplacental unit adds a new dimension to xenobiotic metabolism. The capacity for oxidative drug metabolism and conjugation reactions has been particularly demonstrated in the placenta. Foetal metabolic capacity is generally low until very near to term (Dybing and Soderlund, 1999).

In the first days/weeks of life, as well as in elderly people, the metabolism of toxic compounds can strongly differ from that in healthy adults. In infants four factors have to be taken into account. In the first days following birth the intestinal and blood–brain barriers are not fully developed, thereby allowing a number of substances more readily to be absorbed in the gastrointestinal tract and to reach the central nervous system. The hepatic detoxification reactions (e.g. the glucuronidation of bilirubin) as well as the renal elimination of xenobiotics do not proceed as efficiently as they do later in childhood and in adults.

In elderly people the water content of tissues, the blood flow and the plasma protein binding capacity are reduced, whereas tissue fat content is increased, thereby strongly affecting the distribution of toxic compounds within the body. Furthermore, the renal elimination capacity decreases in the elderly. In cases of severe liver disease the hepatic biotransformation capacity may be reduced.

4.4. Variability in response following different dosing schedules

4.4.1. Peak levels vs time-integrated exposure to toxicants

Dose rates and tissue concentrations are key factors in determining toxicity, as illustrated by the examples mentioned below. Acute exposure to high doses of ethanol results mainly in neurotoxicity, symptoms ranging from gradual reduction of visual acuity, decreased sense of smell and taste, increased pain threshold, impaired muscular co-ordination, nystagmus, staggering gait, nausea and vomiting, diplopia and hypothermia to loss of consciousness and death. In contrast, chronic exposure to intermediate doses of ethanol leads to liver damage (fat accumulation, alcohol hepatitis, cirrhosis).

Depletion of the antioxidant tripeptide glutathione is a frequent cause of steep dose–effect curve, for example with the liver toxicant paracetamol (Rumack and Matthew, 1975). Reaction of glutathione with reactive toxicants usually leads to stable conjugates that, after shortening of the peptide and acetylation, are excreted as mercapturates. As cellular glutathione can be depleted by high concentrations of reactive toxicants but is readily maintained by biosynthesis at lower concentrations, the same total dose that induces toxicity when administered as a single, acute dose may be non-toxic when given over a longer period.

If a metabolic pathway that produces toxic metabolites is readily saturated, as occurs for some P450-dependent oxidations, then administration of a protoxicant in excess of the maximum metabolic rate will not affect their rate of production. If a large part of the excess protoxicant is eliminated unchanged, as is probably the case for many reported studies with benzo[*a*]-pyrene (Gerde et al., 1993), then the excess protoxicant will be ineffective. In turn, toxic responses at lower, more realistic doses where activation of the protoxicant is more efficient, may be underestimated. On the other hand, if the excess dose is stored and later made available to the activating enzymes, the exposure is prolonged and differences in tissue distribution may lead to altered organotropy of the toxicant. An example of storage-prolonged dose occurs for inhaled butadiene, which is metabolised in mice first to the monoepoxide, then to the more mutagenic diepoxide. During inhalation of butadiene, the monoepoxide initially accumulates in fat and is later released, thus prolonging the availability of substrate for the activating step to the diepoxide (Thornton-Manning et al., 1995). In the case of the insecticide DDT, which is known to accumulate in adipose tissue, short-term exposure leads to neurotoxicity, whereas prolonged exposure results in hepatotoxicity (Murphy, 1986).

4.4.2. Extrapolation for different duration of exposure — temporal extrapolation

Temporal extrapolation will only be necessary if a hazard has been identified from an animal study in which the duration of exposure does not match the exposure in humans. For instance, in the case of dietary exposure, this situation is encountered when only subchronic studies in experimental animals are available, since human exposure through food will generally be chronic.

Ideally, the duration of exposure in experimental studies should mimic that occurring in humans. Thus, lifetime administration of a compound to test animals in feed is the most relevant type of study in cases where human exposure is to relatively low dietary concentrations over a long period of time. Accidental high exposures in humans are best mimicked by acute or subacute toxicity studies using high doses.

The scientific basis for the use of chronic studies in experimental animals to mimic lifetime exposure of humans (e.g. a 2-year rodent study as an equivalent to the 70 years or more lifespan of humans) is the concept of physiological time: within an organism, physiological events may be synchronised to an internal timing mechanism which is related to body size. In mammals and birds, virtually all biological times consistently vary with nearly the same body mass exponent (Lindstedt and Calder, 1981). For example, all mammals use approximately the same number of calories per gram of tissue in a lifetime (Lindstedt and Boyce, 1985). However, it should be kept in mind that in some cases (e.g. heavy metals such as cadmium) human lifetime exposure cannot be adequately mimicked in experimental animals (Bhattacharyya et al., 2000).

In cases where temporal extrapolation proves necessary, a number of toxicokinetic and toxicodynamic considerations should be accounted for. For example, compounds that show very slow elimination characteristics and a propensity to bioaccumulate (e.g. TCDD) may not reach steady-state concentrations in shorter-term studies, since steady state is reached at four to five times the elimination half-life. There are other factors that can impact on the predictive value of a shorter-term study as a model for chronic outcomes. These include the reversibility of the toxic effect and the possible presence of repair processes, the slope of the dose–response curve, and the specificity of the toxic outcome. For example, one needs to consider whether an effect seen in a subchronic study would increase in severity with time, or if the nature of the effect would change with time (as is the case with some hepatotoxic agents). Finally, it should be noted that several physiological parameters change with age.

In conducting temporal extrapolation, the basic assumption made is that an effect seen at shorter duration of exposure will also be seen after a chronic exposure, though at lower doses (Dourson et al., 1996). This is a reflection of the general applicability of Haber's Law, which states that the product of concentration and time remains constant.

A number of investigators have examined the ratios of no-observed-adverse-effect levels (NOAELs) and lowest-observed-adverse-effect levels (LOAELs) in subchronic as compared to chronic studies using the same compounds (Vermeire et al., 1999). In general, the average difference between subchronic and chronic values was two to three when geometric means were considered (Weil and McCollister, 1963; McNamara, 1976; Rulis and Hattan, 1985; Nessel et al., 1995; Kalberlah and Schneider, 1998; Pieters et al., 1998). However, the geometric standard deviation ranged from 1.3 to 5.6, since different species, databases with variable sizes, and different data selection criteria were used (Vermeire et al., 1999). For further discussion see section 3.

In conducting such comparisons, a number of factors could have influenced the assessment (Kalberlah and Scheider, 1998; Vermeire et al., 1999). One such factor is dose spacing. For example, in NTP studies, five exposure groups are used for subchronic exposure, but only three in classical 2-year testing. Dose spacing, obviously, affects the precision of the experimentally determined NOAEL/LOAEL; consequently, imprecision in the estimates of individual thresholds will yield imprecise ratios of NOAEL/LOAEL. Also, the depth of

toxicological investigation may vary between chronic and subchronic studies. In the case of "range-finding" studies, for example, only a limited number of potential endpoints are examined. Finally, adaptation processes may occur as a study proceeds.

Overall, it has to be borne in mind that temporal extrapolation is not an easy process, and that a number of factors need to be considered. Knowledge about the mode of action and the toxicokinetics of a compound facilitates the process. However, short-term studies may serve in priority setting. Thus, the lack of hyperplasia in a 90-day study may indicate that the possibility of tumour development at longer exposure duration is less than for a compound with a strong hyperplastic response. Generally, there is a need to be flexible in approaching this problem. Mechanistic considerations should be the guide to deciding upon the most adequate approach.

5. Dose–response considerations

5.1. External vs internal doses

In routine toxicity studies, the dose descriptor generally refers to the amount of chemical administered to the animal per kg body weight (i.e. external dose or intake dose). However, for ingested chemicals there may be a number of reasons why the compound is not able, or only partially able, to pass into the general circulation. The parameter that describes the extent of absorption is the bioavailability, which is defined as the fraction of the dose that is transferred from the site of administration into the general circulation as the parent compound (Renwick, 1993a). Owing to limitations in absorption of a chemical, there will often be a better correlation between the internal dose (or absorbed dose) and observed effects than the external dose and such effects. Extrapolation of toxicity data from animals to humans will be improved if it is based on internal dose rather than external dose (see section 6.4).

There are several reasons why all of the dose of a chemical introduced in the gastrointestinal tract may not be able to pass into the general circulation (Renwick, 1993a). The compound may be too polar to be absorbed completely before it is voided in the faeces. Further, the chemical may be metabolised or it decomposes, owing to the pH, enzymes present in the gut lumen and gut microflora. Also, the agent may be metabolised by the gut wall or liver prior to entering the general circulation. Thus, the content of CYP 3A4 and P-glycoprotein in the small intestine has been shown to influence systemic bioavailability for a number of compounds (Kolars et al., 1991; Thummel et al., 1996; Watkins, 1997). For species differences in uptake, distribution, metabolism and excretion, see section 3.1.

5.2. General issues of dose–response assessment

Assessment of the dose–response relationship is a prerequisite for characterising potential risks from exposure to chemicals, as well as a starting point for safety evaluation and guideline/standard setting. Predicting risks at given or at expected exposure levels can only be conducted if there is adequate knowledge of the dose–response curve. The relative importance of the different parts of the curve, namely the NOAEL, the threshold, the LOAEL, the benchmark dose for a given response (see Edler et al., 2002), and the slope for the critical effect in the most sensitive species, can all be the basis for risk assessment. It should be recognised, however, that animal toxicity studies rarely if ever give the full range of endpoints.

In experimental studies, dosage requirements vary with the aim of the testing. For hazard identification, administration of a maximum dose that can be given without producing irrelevant artefacts is necessary to increase the sensitivity of the experimental model. Quantitative risk assessment and safety evaluation, in contrast, require knowledge of the dose–response relationship including doses that produce no effect (non-active doses) to permit the establishment of a "safe level" for human exposure (Renwick, 1999). In order to address these diverse requirements, toxicity studies should ideally apply a widely spaced dosing scheme, covering a wide range of exposures. The reader is referred to a detailed description of dose–response modelling in the report of Edler et al. (2002).

Two of the important points to address in quantitative assessments of toxic responses to chemical exposures are the following: to decide at which dose levels the pathological reactions starts to become manifest, and to judge whether the responses occurring at the lowest doses are merely a reflection of adaptive physiological reactions or if they are truly adverse (see section 5.3).

Traditionally, the view has been that non-cancer and non-genotoxic cancer endpoints are assumed to express dose thresholds (Lu, 1988; Truhaut, 1991). The highest dose that does not cause any toxicity, the NOAEL, is generally taken as a surrogate for the dose threshold, and is used as the starting point for safety evaluation/risk assessments. The NOAEL may be more precisely defined as the exposure level at which there are no statistically or biologically significant increases in the frequency or severity of adverse effects between the exposed population and its appropriate control; some effects may be produced at this level, but they are not considered as adverse, nor precursors to specific adverse effects. In contrast to chemicals exhibiting apparent dose thresholds, many have expressed the view that genotoxic carcinogens do not express dose thresholds so that all exposures entail some level of risk. In these situations, risk-specific levels of exposure can be defined

for risk assessment purposes with the aid of mathematical models (Younes et al., 1998). In order to imply the existence of a threshold, the underlying mechanism for the adverse effect must not be identical to that of a background process. For all situations where a chemical acts incrementally on a mechanism which has a background rate, a true threshold will not be found (Crump et al., 1976).

For threshold responses, the term no-observed-effect-level (NOEL) has been introduced. This is an exposure level at which there are no statistically or biologically significant increases in the frequency or severity of any effect between the exposed population and its appropriate control. Scientific judgement must be employed in order to decide whether the changes occurring at the lowest exposures are adaptive or adverse. However, the distinction between NOEL and NOAEL is not always made and not always known due to the ambiguity of the biological change.

In assessing hazards from exposure to various substances, differences in sensitivity may lead to misinterpretation of results. Different species and strains of animals may show great variability in toxicological response. For example, the acute toxicity of TCDD varies over several orders of magnitude between hamsters, primates and guinea pigs. This may be due to differences in toxicokinetics, toxicodynamics, or both. In addition, effects may be specific to given species. For example, peroxisome proliferation may be observed in rats, mice and hamsters, but not in humans. Also, the $\alpha_{2\mu}$-microglobulin-associated nephropathy and renal tumorigenicity is confined to the male rat. For more detail see section 3.

5.3. Adaptive vs adverse reactions

An adverse, or "abnormal" effect in toxicological studies has often been defined in terms of a measurement that is outside the "normal" range in treated groups. The "normal" range, in turn, is usually defined on the basis of measured values observed in the untreated control group, and expressed in statistical terms of a range representing 95% confidence limits of the mean. An adverse effect may be defined as a biochemical change, functional impairment, or pathologic lesion that affects the performance of the whole organism, or reduces an organism's ability to respond to an additional environmental change. The initial reactions in the organism following exposure to a chemical agent, may be looked on as being due to a number of different biochemical mechanisms of action, shown in Table 2.

Many of these biochemical mechanisms will at low doses lead to adaptive responses that may be seen as stress reactions to environmental influences whereby the organism attempts to maintain homeostasis. Enzyme induction, changes in hormone levels, and indicators of slightly altered cellular function are examples of such adaptive responses. In many instances, these responses do not lead to clinically significant altered structure or function, namely adverse reactions. However, in many instances it may be difficult to decide whether a response is adaptive or adverse. Enzyme induction, for instance, may in some situations be present as an adaptive response without any biological significance; sometimes it may be beneficial in that it leads to more rapid metabolism and elimination of potentially toxic compounds; or it may be a truly adverse response in that it may lead to increases in reactive intermediates and thus potentiate toxic effects.

The US National Academy of Sciences (1975) defined non-adverse effects as the absence of changes in morphology, growth, development and life span. Furthermore, non-adverse effects do not result in impairment of functional capacity or impairment of the capacity to compensate for additional stress. These effects are reversible following cessation of exposure without detectable impairment of the organism to maintain homeostasis, and do not enhance susceptibility to the deleterious effects of other environmental influences.

On the other hand, adverse effects may be deduced as changes that occur with intermittent or continued exposure and that result in impairment of functional capacity (as determined by anatomical, physiological, and biochemical or behavioural parameters) or in a decrement of the ability to compensate additional stress. Further, adverse effects are irreversible during exposure or following cessation of exposure if such changes cause detectable decrements in the ability of the organism to maintain homeostasis. In addition, adverse effects enhance the susceptibility of the organism to the deleterious effects of other environmental influences (National Academy of Sciences, 1975).

With the refinements of existing, as well as the development of new analytical techniques, more endpoints can be analysed within the same experiment. This will clearly increase the probability of detecting changes; however, it may also increase the difficulty in interpreting the results. With the introduction of novel molecular biological methods to detect subtle changes in gene expression as a result of exposure to xenobiotics, it will be a great challenge to clarify whether such changes

Table 2
Biochemical mechanisms of toxicity

- Induction of specific enzymes
- Inhibition of specific enzymes
- Overload of specific enzymes
- Depletion of protective pools
- Displacement from carrier proteins
- Interaction with endogenous receptors
- Derangement of membrane regulated processes

Modified from IPCS (2000).

simply represent non-adverse alterations of physiological function or if they predict impending development of more serious irreversible injury, should exposure to the chemical continue.

It will often not be readily possible to differentiate between adverse and non-adverse effects from routine toxicity tests. Differentiation between non-adverse and adverse effects requires considerable knowledge of the importance of reversible changes and subtle departures from normal physiology and morphology. Thus, supplementary mechanistic studies may be necessary to determine a NOAEL and to ascertain whether this is different from the NOEL. In the end, the decision has to be based on scientific judgement and expertise.

5.4. Threshold vs non-threshold responses

The issue of dose thresholds is complicated, and can be discussed from a biological and from a quantitative perspective. In this section, thresholds are discussed from a biological perspective, using biological arguments that make the notion of thresholds plausible in a qualitative sense, for example a threshold should be seen as a certain dose range, where a substantial change in response may occur. Below this dose range, no biologically significant effects are expected to occur. From a quantitative point of view a single dose threshold in the strict sense that one molecule may change the response from zero to non-zero, is hard to defend (see Slob, 1999; Edler et al., 2002). Physiological responses in the mammalian organism are well regulated by homeostatic mechanisms in order to maintain cellular equilibrium and normal function. Examples of this are hormonal and electrolyte regulation within the organism. Perturbation of such processes by exogenous chemical exposures may disturb the equilibrium given that the homeostatic controls are surpassed. At low levels of chemical exposure, there is enough plasticity in the various cellular systems so that homeostatic mechanisms will be able to counteract any response initiated by the xenobiotic exposure. Up to a certain degree of occupancy of crucial sites in cellular macromolecules or of interactions with low molecular weight cellular substances, there will not be cellular responses leading to structural or functional changes. Thus, below specific dose thresholds, biological responses will not become evident and the probability of an individual responding is zero. The ability of the body to handle a chemical without toxicity becoming manifest may vary among individuals and across species. Such a threshold level will also be influenced by a number of endogenous factors including age, gender, weight, genetics, nutritional status and disease, and by extrinsic factors such as nutritional composition, smoking and exposure to other chemicals. As such variabilities exist, one will always have individuals within a population who are relatively sensitive and are therefore at increased risk to exposure to some chemicals. Conversely, there are others who are resistant and who require relatively greater exposure to elicit similar responses.

There are numerous examples of acute chemical exposures demonstrating dose thresholds for toxic responses. For instance, hepatotoxic actions by reactive metabolites of drugs such as paracetamol are counteracted at low levels of exposure by the protective actions of cellular glutathione (see section 4.4.1). Another example is methaemoglobin formation by nitrite. Under normal physiological conditions FeII-haemoglobin in the erythrocyte is continuously undergoing oxidation to FeIII-haemoglobin, but this is reduced back to the FeII form by methaemoglobin reductases.

Under physiological conditions, as well as at low levels of nitrite exposure, the circulating levels of methaemoglobin are maintained at low levels and normal oxygen transport is sustained. When the dose level of the nitrite is increased, the reducing capacity of methaemoglobin reductase is surpassed, leading to toxic levels of methaemoglobin resulting in decreased oxygen transport and cyanosis. This latter example illustrates that a biologically plausible threshold should be seen as a small range of doses where the response may change substantially, and not as an exact single dose level. This latter would contradict the fundamentals of enzyme kinetics.

Chronic exposures at levels that do not lead to toxic responses after single doses may still result in damage to the organism due to accumulation of the individual doses in the body and/or an accumulation of cellular interactions. However, the accumulated body burden and/or the accumulated cellular changes must surpass a critical level not counteracted by homeostatic mechanisms, in order to induce a toxic response. Thus, also chronic exposures that are small enough to be controlled by homeostatic processes, will exhibit dose thresholds. An example of this is nephrotoxicity caused by the heavy metal cadmium. The renal cadmium toxicity seen after long-term exposure is dependent on the availability of free ionic cadmium in the tubular cell that is not sequestered by the protein metallothionein synthesised by the kidney. Cadmium induces metallothionein to which it is bound. The complex is filtered in the kidneys through the glomeruli into the primary urine. Thereafter, it is reabsorbed in the proximal tubular cells, where the cadmium–metallothionein bond is broken. The unbound cadmium, in turn, stimulates the production of additional metallothionein. This will then bind the cadmium that is present in the tubular cells, preventing toxic effects of the metal. Thus, the kidneys (and similarly the liver) act as cadmium sequestration stores. However, if the metallothionein-producing capacity is exceeded, damage to the proximal tubular cells occurs. Most animal data indicate that at a renal

cortex level of 100–150 mg Cd/kg tissue must be reached before renal tubular damage will occur.

Lipophilic compounds, such as dioxins and polychlorinated biphenyls (PCB), are stored in body fat, from which they may be released slowly. The kinetics of release to other tissues, in addition to the total body burden, will determine the toxicity of such compounds.

A special situation with chronic exposures may be envisaged when the chemical or its metabolite reveal genotoxic properties. In analogy with carcinogenic responses from ionising radiation, many have viewed genotoxic responses by chemicals to be stochastic, so that all interactions with the genetic material in the body entail some degree of risk. However, homeostatic arguments can also be brought forward regarding genotoxic responses. At low levels, reactive chemical molecules may preferentially be detoxified by metabolic reactions or low molecular cellular constituents such as thiols and antioxidants. Also, permanent DNA modifications may be restored by repair processes. Further, initiated cells may be removed by apoptotic reactions. As the doses inducing cancer development have been shown to be related to the time function in a log–log linear fashion (Druckrey, 1967), it follows that, at least theoretically, there should be a practical dose threshold dictated by the longevity of the species. However, up until now it has not been possible with the existing methodology and logistics to determine whether genotoxic chemicals exhibit dose thresholds or not. The classical ''mega-mouse'' carcinogenicity experiment with 2-acetylaminofluorene, revealed a linear, non-threshold dose response for liver tumours, whereas for urinary bladder tumours there was evidence for a dose threshold (Littlefield et al., 1979). Also, theoretically, it will not be possible to prove the absence of a dose threshold for genotoxic responses since one can never prove a negative. The traditional position advocated by many with respect to risk assessment of genotoxic compounds has therefore been to view these chemicals as not to exhibit dose thresholds for their responses. The non-threshold concept for risk assessment of genotoxic compounds has recently been challenged (Purchase and Auton, 1995).

5.5. Hormesis

A considerable body of literature supports the assertion that low doses of otherwise toxic compounds have beneficial effects under specific and limited conditions, a concept termed hormesis, exhibiting U-shaped dose–response relationships (Calabrese and Baldwin, 1998). Chemical hormesis was judged to have occurred in approximately 350 of 4000 evaluated studies (Calabrese and Baldwin, 1998). Growth responses were the most prevalent endpoints showing a hormetic response, followed by metabolic effects, longevity, reproductive responses and survival. The average low-dose maximum stimulation was approximately 50% greater than controls. The hormetic dose–response range was generally limited to about one order of magnitude below the NOAEL. If a U-shaped dose response is apparent, this finding has to be ascertained as to whether there are no other shapes that could explain the data with similar goodness of fit, which may be difficult if only one or two doses document the U-shape. However, at present there is no consensus that the hormesis concept has general applicability in hazard characterisation of low molecular weight chemicals.

5.6. High-to-low dose extrapolation

One critical issue is the selection of the top dose in repeated dose toxicity tests. Traditionally, the need for long-term studies to detect carcinogenic effects in a small number of animals has led to the general introduction of the so-called maximum tolerated dose (MTD) as the standard high dose. The default MTD is the dose that produces a 10% decrement in growth and body weight gain in the absence of other toxic manifestations (Swenberg, 1995). The relevance of the MTD has been questioned, however, given the potential of such high doses to interfere with physiological parameters (e.g. by saturating xenobiotic metabolising enzymes and/or by blocking detoxification pathways), to produce nutritional imbalance, or to produce spurious effects with no relevance to human exposures (Counts and Goodman, 1995; Renwick, 1999).

In extrapolating from high to low doses, a number of problems may be encountered, which affect the toxicokinetics and/or the toxicodynamics of the test compound. Among these problems, nutritional effects may be observed. These include changes in the palatability of the diet in the presence of high concentrations of the test compound, as well as the potential for high dietary concentrations of a compound which is not a nutrient per se to interfere with the digestion or absorption of normal nutrients (Renwick, 1999). Other effects to consider are of biochemical (e.g. inhibition or induction of enzymes, depletion of cofactors) or physiological nature (e.g. interference with hormonal balance, alteration of normal organ or cellular functions) (Marrs, 1993; Morgenroth, 1993; Poulsen, 1993; Renwick, 1999).

Generally, the default assumption in extrapolating from high to low doses is that a linear relationship exists between dosage and target organ exposure to the active chemical entity. However, non-linearity might occur both with respect to toxicokinetics and toxicodynamics. In the case of toxicodynamics, even the mechanism of action may differ between high-dose and low-dose exposures. Non-linear toxicokinetics may be encountered at various levels: absorption may be influenced if

an active transport mechanism is involved and becomes saturated at given doses. It may also be altered due to the saturation of binding of the chemical or its active metabolite(s) to plasma or tissue proteins, or if, through a decrease in cardiac output at high doses, the rate of tissue distribution and clearance is decreased. Metabolism, finally, can be inhibited at high doses through saturation of metabolising enzymes by the substrate, through inhibition by the end-product, or through depletion of necessary cofactors. Also, it is possible that at higher doses an alternative pathway of metabolism is used which produces a (toxic) metabolite that is not normally generated (or generated at much lower levels) at lower doses (Renwick, 1999). An example of this phenomenon (dichloromethane) is presented in section 5.3. In fact, examination of the shape of carcinogenesis dose–response curves of 315 chemicals tested in the National Cancer Institute–National Toxicology Program revealed that data were more often consistent with a quadratic response than with a linear response. Also, genotoxic compounds differed from linearity more often than non-genotoxic compounds (Hoel and Portier, 1994).

Mathematical modelling of dose–response relationships would improve the hazard characterisation process, and mode of action data can be used to extend a PBTK model into a full biologically-based dose–response model (see Edler et al., 2002).

In some instances, where a LOAEL, but no NOAEL, could be determined for a threshold effect, it might be necessary to extrapolate from the LOAEL to an expected NOAEL (e.g. by introducing an uncertainty factor). Traditionally, uncertainty factors of 3–10 have been used by different agencies for this purpose. Ratios of LOAELs to the respective NOAELs (in the same studies) of either subchronic or chronic exposures were analysed by a number of authors. Dourson and Stara (1983) examined data from Weil and McCollister (1963), and found that 96% of the ratios between LOAELs and the NOAELs for the same endpoint in the same study had values of 5 or less. Kadry et al. (1995) evaluated similar ratios for chlorinated compounds. 91% revealed a factor of 6 or less, and all ratios were 10 or less. Given the typical dose spacing in most toxicological studies, it should be borne in mind that the real NOAELs are often higher than those established in guideline-type testing. An advantage in comparison to using the NOAEL may be to use all of the available dose response information to derive a benchmark dose as a starting point, or to apply other models for low dose extrapolation (Kalberlah and Schneider, 1998). It should be kept in mind that the ratio of LOAEL to NOAEL has to account for the fact that this ratio is strongly determined by the dose design of the study and may simply reflect the dose spacing typically used in in vivo toxicity studies (see Edler et al., 2002). Also, the number of animals in the experimental groups will affect the precision of determining the NOAEL/LOAEL.

Traditionally, the shape of the dose–response curve is not used to derive a NOAEL and thus in safety evaluation. Apart from ensuring that the number and spacing of data points is adequate to provide a reasonable estimate of the NOAEL, all other data points are often ignored. However, the slope of the dose–response curve may be examined to provide a general impression of the steepness of response. Modern methodologies, however, allow for better use of all data points, and provide means to reduce uncertainties around the NOAEL determined experimentally. In the benchmark approach a regression function fitted on the response data is used to estimate the dose at which adverse effects start to arise. In the benchmark concept, one needs to postulate a critical effect size (CES) below which there is no reason for concern and the associated critical effect dose (CED) at which the average animal shows the critical effect size defined for the particular endpoint (Slob and Pieters, 1998). A major advantage of the benchmark approach is that all of the experimental data points are used in extrapolation and that there is no need to extrapolate from a LOAEL to a NOAEL. However, a meaningful application of the benchmark approach requires at least three dose groups showing different response levels, but preferably more (see Edler et al., 2002).

5.7. Current situation and future prospects

The test methods that are employed in hazard identification and hazard characterisation, have been developed over a 40–50-year period. This has generated many background control data to put results into context. Most laboratories have good procedures for minimising and recognising the presence of extraneous factors such as infection and nutritional imbalance, which could compromise the results. The use of good laboratory practice (GLP) provides a high level of assurance of the validity of the studies and the results. However, the major problem comes with the descriptive nature of the methods applied and the relatively small group sizes studied. The investigator is dependent on assessing the difference between control animals and treated animals, which is influenced by biological variability. The detection of a low incidence effect or the derivation of a NOAEL can be problematical and can be materially affected by the sensitivity of the test method, the selection of dose levels and number of animals studied.

The major potential advances will come with wider application of the understanding of underlying mechanisms in the aetiology of adverse effects. An understanding of mechanisms will allow for better interpretation and extrapolation of results. There is also

considerable potential in being able to replace descriptive studies that are demanding in terms of conduct time, laboratory animals and resources with considerably quicker and potentially more accurate studies which determine whether particular mechanisms of action apply.

It is expected that the application of molecular biological methods for the identification of molecular targets and delineation of molecular mechanisms of action will markedly improve hazard identification and characterisation in the future. The use of novel methods in genomics and proteomics will greatly expand the database related to interactions of chemicals with biological systems. However, given the redundancies and homeostatic controls in biological systems, it will be important to sort out the insignificant responses from the critical events revealed in genomics and proteomics studies. For toxicological hazard characterisation, it will be essential to verify or refute findings with genomics and proteomics methods in integrated, whole organism systems. Further developments in application of non-radiolabelled technology such as nuclear magnetic resonance (NMR) spectroscopy, will presumably increase the understanding of toxicokinetics and thus improve the process of hazard characterisation.

In the future it may not be necessary to have to demonstrate that a particular event, such as the formation of a tumour, has occurred after prolonged dosing to be aware that the compound has the potential to do this and also to define the dose–response curve. Of equal importance will be the ability to determine that a compound does not have the significant potential to cause such an effect with dosing an animal for a long period of time. This type of approach identifying an early, critical event has been termed the surrogate marker approach and it offers the potential for a reduction in time, use of animals and cost, and an increase in the quality and quantity of the predictions that can be made.

The demand for the demonstration of the safety of an ever-widening group of food chemicals (e.g. natural flavourings, food-contact articles) will require a reliable means of assessing priorities. It is inconceivable to achieve this by using an in-depth toxicological test program. Rather, priorities will need to be determined on the basis of reliable assessment of actual exposure levels and consideration of physical-chemical properties. The underlying premise to support such a strategy is that a common exposure level can be defined that will not cause any significant adverse effect for any chemical regardless of its chemical class. This exposure level is termed the "threshold of toxicological concern" (TTC) (see Kroes et al., 2000; Edler et al. 2002). The concept is widely accepted by toxicologists and is used by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) for flavours, and by the US Food and Drug Administration (FDA) for food-contact materials. However, there is ongoing debate about the actual level at which the TTC value should be set.

6. Species differences and interspecies extrapolation

6.1. Introduction

In general, animal models are good predictors of toxic outcomes in humans, at least from a qualitative standpoint. However, species differences in toxicity are well known, both of a quantitative as well as a qualitative nature. There are a number of causes for observed differences in target organ sensitivity and selectivity towards chemicals. In principle, such differences may be due to qualitative or quantitative variability in toxicokinetic (the delivery of the chemical to the site of action) or toxicodynamic factors (the inherent sensitivity of the site of action to the chemical) (Table 3). (See also section 3 for a general discussion of causes underlying variability in toxic responses.)

Numerous differences between experimental animals and humans contribute to the uncertainty around the use of animal toxicity data for human risk assessment. Because of these differences and uncertainties, appropriate and reliable human data, whenever available, should take precedence over data from animal studies.

Anatomical and physiological differences exist between species (see section 2 — Causes underlying variability in toxic responses). Several of these factors contribute to species differences in toxicokinetics. One important source of such differences is metabolism: small animals tend to metabolise chemicals more rapidly than humans because their relative liver weight is pre-

Table 3
Principal types of species differences in chemical toxicity

I. Qualitative differences
Species-specific differences in pathogenetic mechanisms
II. Quantitative differences
A. Differences in kinetic factors
Differences in chemical delivery leading to differences in target dose
B. Differences in dynamic factors
Differences in target sensitivity related to, for example, receptor level and affinity, cell proliferation, apoptosis, tissue repair

sumably greater, liver perfusion is higher, and the activity of most mammalian hepatic metabolising enzymes increases relatively with decreasing body weight (Krasovskii, 1976). The most important cause of species differences in susceptibility to chemical toxicity seems to be differential biotransformation (Walker, 1978). Species variabilities in biotransformation occur either as species-specific deficiency or limitation in a particular metabolic reaction, or as variabilities in activity and balance of competing metabolic reactions (Caldwell, 1981).

Sources of interspecies differences in toxicodynamics include:

- anatomical differences, for example the effect may occur in an organ of questionable relevance to humans (e.g. the rodent forestomach);
- physiological differences, for example the hormonal control of the target organ may differ between species;
- biochemical differences, for example the balance of bioactivation/cytoprotection may be different or a key biochemical component may show species differences (e.g. $\alpha_{2\mu}$-globulin nephropathy) (Borghoff et al., 1990; Flamm and Lehman-McKeeman, 1991; Hard et al., 1993).

Differences in toxicodynamics may lead to quantitative differences in response, but, in some cases may result in the conclusion that effects detected in experimental animals are of no relevance to humans.

6.2. Examples of species-specific toxicity

Usually, species differences in toxicity are of a quantitative, and not a qualitative nature. However, there are examples were human effects are predicted from laboratory species, but not observed in humans (Table 4).

In addition, non-reactive agents such as butylated hydroxyanisole, which only produce tumours in the rodent forestomach after prolonged treatment, may be of little relevance to humans (IARC, 2002).

Conversely, there are several historical examples of failures in laboratory animals to predict human toxicity, including leukaemia from occupational exposure to benzene and skin, eye and intestinal lesions caused by the beta-adrenergic blocking drug practolol.

Table 4
Some effects detected in laboratory species but not observed in man

Agent	Toxic outcome	Species
Acetylsalicylic acid	Foetal abnormalities	Rat
Phenobarbital	Liver tumours	Mouse and rat
Oral contraceptives	Bleaching of the retina	Monkey
Penicillin	Rapidly fatal	Guinea pig
Saccharin	Bladder tumours	Rat
Clofibrate	Liver tumours	Rat

Some carcinogens show species specificity in that effects determined in animal experiments are not relevant for humans. Examples of this are some non-genotoxic renal carcinogens which are only active in the male rat (Borghoff et al., 1990; Flamm and Lehman-McKeeman, 1991; Hard et al., 1993). Compounds such as 1,4-dichlorobenzene, dimethyl methylphosphonate, hexachloroethane, isophorone, *d*-limonene and tetrachloroethylene cause hyaline droplet nephropathy which leads to replicative tubule cell proliferation, and ultimately renal cell tumours via reversible interactions with the male rat-specific urinary protein $\alpha_{2\mu}$-globulin.

Another example of a species-specific carcinogenic response is the bladder tumours seen in rats after administration of sodium salts. Amorphous calcium phosphate precipitates of a number of sodium salts, including sodium saccharin, ascorbate, glutamate, bicarbonate and chloride, are cytotoxic to the rat urothelium and generate a mild regenerative hyperplasia which subsequently develops into bladder tumours (Cohen, 1999). These effects are greater in male than in female rats and appear not to occur in mice, hamsters or monkeys (Ellwein and Cohen, 1990; Cohen, 1995; Cohen and Lawson, 1995). The underlying cause is a combination of urinary pH $\geqslant 6.5$, high concentrations of calcium phosphate and protein, and high osmolality critical for formation of precipitates which is unique to the rat. Other agents (e.g. melamine), which cause urinary bladder tumours as a secondary response to urinary calculus formation, could conceivably cause a similar response in humans given that exposures are high enough to generate calculi (IARC, 1999a).

Long-term administration of sulfonamides to rats and mice leads to alterations in thyroid hormone homeostasis with increased thyroid stimulating hormone (TSH) secretion and ultimately thyroid follicular cell tumour development. Sulfamethazine and sulfamethoxazole do not affect thyroid hormone homeostasis in monkeys due to insensitivity of monkey thyroid peroxidase (McClain, 1985; Takayama et al., 1986). Sulfonamides are not considered to represent a carcinogenic hazard to humans (IARC, 2001).

The plasticiser di(2-diethylhexyl) phthalate (DEHP) is a low-level contaminant of many foodstuffs. Some of the effects of DEHP in experimental animals are judged to be not relevant for humans, whereas other are (CSTEE, 1998). DEHP produces liver tumours in rats and mice by a non-DNA-reactive mechanism involving peroxisome proliferation. Hepatic peroxisome proliferation depends on a nuclear receptor, PPARα, to mediate this response. Oral administration of DEHP failed to elicit markers of peroxisome proliferation in PPARα-deficient mice (Ward et al., 1998). Differences between responsive rodents and humans in PPARα-mediated regulation of gene expression are consistent with the lack of activity of DEHP metabolites in human

hepatocyte cultures and in the liver of DEHP-exposed non-human primates. Therefore, the mechanism of hepatocellular tumours in rats and mice is not relevant to humans (IARC, 2000). However, DEHP elicits testicular and kidney toxicity in PPARα knockout mice at a dose which caused peroxisome proliferation in wild-type animals (Ward et al., 1998), indicating that these effects are relevant for humans.

6.3. Species differences in toxicokinetics and toxicodynamics

Quantitative species differences in metabolism are a rule, rather than an exception. In addition, there are some well-known examples of major species deficiencies (Table 5).

There are many quantitative species differences in metabolism (Caldwell, 1980). Such differences will in many instances influence toxicity. Indeed, such differences in sensitivity have been exploited in organophosphorus pesticides such as malathion, which are much more toxic to insects than to human and other mammals. A classic example is the drug hexobarbitone, which shows striking metabolic differences between species, correlating with the pharmacological effect (Table 6).

Long-term inhalation exposure to methylene chloride (dichloromethane) produces increased incidences of benign and malignant lung and liver tumors in mice, but not in rats or hamsters. Mechanistic studies have established a link between GST-mediated metabolism of methylene chloride and its carcinogenicity in mice. The GSTT1 responsible for metabolism and genotoxicity of methylene chloride is expressed to significantly greater extents in mouse lung and liver than in the corresponding rat, hamster and human tissues. The available data suggest a plausible mechanism for the development of liver and lung tumours which occur in mice, but not in rats or hamsters exposed to methylene chloride (IARC, 1999b). The cancer risk of methylene chloride to humans is overestimated by about two orders of magnitude, if toxicokinetic differences between mice and humans are not taken into account (Andersen et al., 1987). Further, GSTT1 is polymorphic in humans and the frequency of the GSTT1 homozygous null genotype ranges from 10 to 60% in different ethnic and racial populations around the world. Human risk estimates for methylene chloride carcinogenicity are 23–30% higher when the GSTT1 polymorphism is not included in the models (El-Masri et al., 1999).

With respect to acute toxicity, potency usually varies little across mammalian species, although for a small number of compounds LD_{50} values may vary by one to two orders of magnitude. On the other hand, many carcinogens show quantitative differences in activity between species. A classic example is 2-naphthylamine, which appears to be much more potent in humans and dogs than in rats (Kriek, 1969; Radomski, 1979; Gold et al., 1984). Rats, on the other hand, are much more susceptible towards the hepatocarcinogenicity of 2-acetylaminofluorene than mice or hamsters, while guinea pigs and monkeys appear to be resistant (Miller et al., 1964; Dyer et al., 1966; Miller, 1970; Gold et al., 1984). Aflatoxin B_1 is a very potent liver carcinogen in rats but is less potent in monkeys and hamsters and essentially non-carcinogenic in feeding experiments in mice (Herrold, 1969; Wogan, 1973; Gold et al., 1984; Thorgeirsson et al., 1994). Chemicals causing thyroid tumours

Table 5
Species deficiencies in metabolism of foreign compounds

Species	Compounds	Deficient reaction	Outcome
Cat	Small phenolic compounds	Glucuronidation	Sulfate rapidly depleted, leading to increased toxicity
Cat	Small aromatic acids	Glucuronidation	Glycine rapidly depleted, leading to increased toxicity
Dog	Many primary amino groups	Acetylation	Failure to conjugate exacerbates toxicity
Guinea pig	Aromatic amines	*N*-Hydroxylation	Results in increased toxicity in some cases, but protects in others
Rat	Aliphatic amines	*N*-Hydroxylation	Generally increased toxicity

Table 6
Species differences in the oxidation and duration of action of hexobarbitone[a]

Species	Duration of action (min)	Plasma half-life (min)	Relative enzyme activity (μg/g/h)	Plasma level on awakening (μg/ml)
Mouse	12	19	598	89
Rabbit	49	60	196	57
Rat	90	140	135	64
Dog	315	260	36	19

After Quinn et al. (1958).
[a] Same dose in mg/kg body weight.

through an indirect mechanism via sustained elevation of TSH levels are presumably much more potent in rats than in humans, due to the large differences in thyroid physiology between the two species (IARC, 1999a, 2001, 2002). In general, however, the quantitative differences in carcinogenic activity between animals are not large. Differences in potency between rats and mice are within a factor of 10 for 74% and within a factor for 100 for 98% of known carcinogens, respectively (Gaylor and Chen, 1986; Gold et al., 1989).

For compounds that mediate their toxicity via an initial interaction with specific tissue receptor, the receptor-binding affinity of such compounds often correlates with its potency in eliciting a biological response. The intracellular "Ah receptor" or "dioxin receptor" binds and mediates the response to TCDD and other aromatic compounds. In general, the rank order binding affinity of TCDD and related chemicals to the Ah receptor has been demonstrated to be similar to their rank order potency to elicit a broad spectrum of biochemical, morphologic, immunologic, neoplastic, developmental and other reproductive effects (Poland and Knutson, 1982; Safe, 1986). However, not all aspects of species and strain differences in toxicity of dioxins is correlated with Ah receptor binding affinity. For instance, there is approximately a 1000-fold difference in sensitivity to the acute lethal effects of TCDD between Long–Evans and Han/Wistar rats, yet there are no major differences between Ah receptor binding affinity (Pohjanvirta et al., 1999).

6.4. Interspecies extrapolation (animal to man)

A number of approaches have been used to extrapolate between species. Ideally, adverse effects in one species would be used to predict hazards to another species based on:

- comparison of the levels and persistence of the chemical (and/or its biologically active products at the critical tissue site);
- demonstration that the toxicodynamic process is likely to be comparable.

In order to extrapolate toxicity data from animals to humans, this has to account for three aspects:

- dose normalisation or scaling to allow for the differences in body size, which is frequently done simply by expressing the dose in mg/kg body weight or based on body surface area;
- the toxicokinetics of the compound, especially any metabolic bioactivation processes and differences in first-pass metabolism. In some cases, the dose delivered to the target organ can be derived by PBTK modelling which can allow for species differences in the blood flow to various organs and the extraction by specific organs, as well as rates of absorption and elimination (Gerlowski and Jain, 1983);
- the nature and sensitivity of the target organ toxicity (toxicodynamics) (Monro, 1993).

In the absence of information on species differences in toxicokinetics and toxicodynamics, the default position will be to extrapolate toxicity data generated in rodents directly to humans on a mg/kg body weight basis.

Different types of dose normalisation or dose scaling techniques can be used. Dose scaling based on physical characteristics (e.g. body weight, body surface area, caloric requirements) is the most frequently used type of approach. Among such approaches, scaling on the basis of body weight (isometric scaling) is most often applied in toxicology (Mordenti, 1985; Ings, 1990). Average daily doses in animals, expressed as mg/kg body weight, are normally used as a basis for risk assessment. The basic assumption is that numerous biological parameters show a linear correlation with body weight (Davidson et al., 1986). Quantitative interspecies differences in physiological processes, organ perfusion and clearance also influence interspecies relationships based on body weight (Renwick, 1991, 1993b).

Other factors, including absorption, plasma protein binding, and biliary excretion are independent of body weight (Mordenti, 1986). As several of these functions correlate with body surface area, the latter was also used as a basis for interspecies extrapolation (allometric scaling). Calabrese et al. (1992) assumed that body surface area equals body weight$^{0.67}$. The larger the test animal species, the smaller the conversion factor. It should be noted, however, that the use of body surface area may introduce inaccuracies in extrapolation, particularly since the specific metabolic profile of a compound may not correlate with the overall metabolic rate of the animal and, hence, with the surface area (Lu, 1985).

An alternative allometric scaling method to correct the intake/exposure is scaling based on caloric demand. The basic assumption here is the similarity in basal metabolic rates across different species. To apply this method, body weight$^{0.75}$ is used. As this approach allows for species differences in many physiological functions, interspecies variabilities in toxicokinetics will have been accounted for (Kalberlah and Schneider, 1998; Vermeire et al., 1999). Allometric scaling, be it based on body surface area or caloric demand, is more closely related to internal concentrations of a chemical rather than to the external dose. Hence, it may give a better estimate of interspecies differences in target organ dose and toxic effect (Kalberlah and Schneider, 1998).

Apart from dose scaling on the basis of physical characteristics, two other approaches have been employed:

- Functional activity (lifespan): scaling exposure on the basis of lifespan is relevant to the extrapolation of long-term cancer assays in animals and humans.
- Multiple species regression: this approach requires conventionally adequate data from at least four species in order to estimate the equivalent dose for humans (Ings, 1990). This quantity of data, however, is seldom available.

Ideally, the choice of the dose metric for interspecies extrapolation (dose scaling) should be mechanistically driven. Knowledge of compound characteristics may allow for the deviation the use of standard approaches related to daily doses. In the case of dioxins, for example, body burden was used as the basis for extrapolation from animal studies to human exposure (WHO, 2000). Equivalent body burdens were used to calculate the human daily intake corresponding to the toxic effect observed in animals.

7. Hazard characterisation of chemical mixtures

7.1. Introduction

Usually, risk assessment of food components addresses human health risk based on in vivo toxicity data generated from exposures to individual chemicals. Also, application of uncertainty factors to allow for intraspecies and interindividual variability generally does not take into account the possibility of combined exposures. However, food can be considered as an extremely complex and variable chemical mixture estimated to consist of several thousands of chemicals (nutrients, low molecular weight chemicals and secondary plant metabolites). Clearly, in principle, every class of food chemicals may exhibit joint similar or joint dissimilar action, leading to non-interactive combined effects, and may also interact with one another, altering the degree and maybe also the nature of the potential toxic effects of individual food chemicals. Therefore, the question has been raised whether such potential combined or interactive adverse effects from exposure to food chemicals are likely or unlikely to occur in humans. Given the likelihood of joint action and the current state of the art of mixture research this section will mainly address this question for low molecular weight chemicals and put these findings into the perspective to our current understanding on the behaviour of nutrients.

7.2. Mixture terminology

Three basic concepts of joint action or interaction of combination of chemicals have been defined by mathematicians, and these are still valid today in the field combination toxicology (Bliss, 1939; Cassee et al., 1998). The first concept is called *simple similar action*, which is also known as simple joint action, or dose addition. This is a non-interactive process because each of the chemicals in the mixture acts in the same way, by the same mechanism(s), and differs only in their potencies. Simple joint action allows the additive effect to be described mathematically, using the summation of the doses of the individual compounds in a mixture, after adjustment for the differences in potencies. This form of action also serves as the basis for the use of toxic equivalency factors used to described the combined toxicity of isomers or structural analogues such as dioxins.

The second concept of joint action is called *simple dissimilar action*, which is also referred to as simple independent action, independent joint action, or response- or effect-addition. The modes of action and possibly the nature and site of action differ among the chemicals in the mixture which exert their individual effects, but do not modulate the effect of other constituents of the mixture. Effect addition is the additive effect determined by the summation of the effects to each compound in the mixture. The term ''response addition'' should be used more specifically to describe the ''number of responders'' in a population. This holds true if each individual of the population has a certain tolerance to the chemicals of the mixture and the individual will only exhibit a response to a toxicant if the concentration exceeds the tolerance dose. In this case, the number of responders will be given rather than the average effect of a mixture on a group of individuals.

The third concept is called *interaction*, and it describes the combined effect between two chemicals resulting in a stronger effect (synergism, potentiation, supra-additivity) or weaker effect (antagonism, inhibition, sub-additivity) than expected on the basis of either dose or response additivity. The term ''interaction'' should not be viewed in the physiological sense to describe a biological interaction with a target or receptor, but as an empirical description to characterise departure from additivity. The mechanism behind the ''interactions'' may be of physicochemical and/or biological nature and an interaction might occur in the toxicokinetic phase (uptake, distribution, metabolism and excretion of the chemical) and/or in the toxicodynamic phase (interaction of the chemical with the tissue target). Situations can occur in which each chemical affects a process at a different site. Under such circumstances a weak effect by one chemical could be amplified by the weak action of the second substance at a subsequent step in the process, such that the combined effect would exceed that of either compound alone (greater than effect additivity).

Concerns about possible joint actions and interactions between food chemicals relate to dose-additivity and synergism (or supra-additivity), because interactions

which reduce toxicity would have fewer possible health implications on the mixture. Dose additivity could lead to health concerns when the intakes of each individual compound in a mixture are at levels below their adverse effect level, but the combined intake of the compound results in an effect level (i.e. above the NOAEL of the individual compounds). In contrast, an interaction would usually require each additive to be present in sufficient quantities to produce an effect, so that one action can be modified by the action of the other compound.

7.3. Current status of mixture research and implications for food chemicals

Toxicity studies with defined chemical mixtures have shown that the type of combined action or interaction found at clearly-toxic-effect levels does not predict what will happen at no-toxic-effect levels, including levels only slightly lower than the LOAEL (see review of Cassee et al., 1998; Groten et al., 2000). Even if one of the chemicals occurs at a slightly-toxic-effect level the type of combined action of the mixture may be different from that occurring at clearly-toxic-effect levels. However, precisely what happens at no-toxic-effect levels (including exposure levels only slightly lower than the LOAEL) is what counts in assessing the potential health risk of humans exposed to mixtures of these chemicals. Obviously, as soon as the exposure levels of the chemicals in the mixture are in the range of NOAELs, no additivity and no potentiating interaction has been found, indicating the applicability of the basic concept of "independent joint action" (Cassee et al., 1996a; Groten et al., 1997; see also section 4). On the other hand, several other in vivo studies with chemicals that show the same target organ and the same mode of action have revealed that the toxicity of a mixture of similarly-acting toxicants, even at levels slightly below the LOAEL of the individual compounds, corresponded to the effect expected on the basis of the additivity assumption (Jonker et al., 1996; Cassee et al., 1996b; Tajima et al., 2002). In these cases the "dose addition" model represents the basic concept to be used for hazard assessment. This model is applicable over the whole range of exposure levels from low non-toxic levels to NOAELs.

From the limited number of toxicity studies as described above, it appears that in most cases exposure to mixtures of chemicals at (low) non-toxic doses of the individual chemicals in the mixture is of no health concern, and the probability of increased health hazard due to additivity or potentiating interaction seems to be small, since the dose of chemicals to which humans are exposed is generally much lower than the NOAEL. Exceptions to these rules may be mixtures of chemicals with a similar mode of action or with evidence of potentiating interaction, and mixtures with no or very small margins of safety.

To account for dose additivity JECFA, for instance, acknowledges that some food additives share common properties, and these are allocated a "group-ADI". Additives which share close structural similarities may be grouped together for one of two reasons. A group ADI (acceptable daily intake) may be allocated when each member of the group is metabolised to a common metabolite, the activity of which determines the toxicity profile and hence the NOAEL. Since any member, or combination of members, of the group could give rise to a potentially toxic dose of the metabolite, the NOAEL and ADI could be based on any member of the group (using molar equivalents of the toxic metabolite formed). The ADI would then apply to all members of the group, and the total combined intake should not exceed the ADI (dose-additivity). An excellent example of this is the series of esters of allyl alcohol, which are used as flavours and which are hepatotoxic in animal studies due to their conversion to allyl alcohol. The ADI applies to the total combined intake for allyl esters. Simple dose-additivity would apply, because each compound in the combination would produce the same metabolite. Alternatively, additives would be considered as a class when they showed a common effect, or a common mechanism/mode of action, despite not sharing a common metabolite. Potentiating interaction is expected to be only of minor importance in view of the low intake levels of non-nutritive food components.

A different situation exists for nutrients with their relatively small margins of safety (i.e. small margin between recommended daily allowances and the minimum toxic dose). Nutritional imbalance may result in deficiencies but also in exceeding the margin of safety. Nutrients are biologically active, in contrast to additives that are preferably as biologically inert as possible, which may explain the relatively small margin of safety for nutrients. Thus, for risk assessment purposes, the priority category of food chemicals would seem to be the nutrients with their small margins of safety. However, the mixture of nutrients is necessary for growth, maintenance and reproduction of humans, and when in balance (a balanced diet) the mixture as such is a prerequisite rather than a threat to human health. It is important to be on the alert for interactions of non-nutritive and non-food chemicals with nutrients, leading to nutritional deficiencies. On the other hand, one should also consider the possibility of potentiating interactions between non-nutritive, or non-food (e.g. drugs, natural toxins) components and nutrients.

Finally, the mixture of nutrients is an excellent example to illustrate that application of the dose addition concept (as a default concept) for assessing the health risks of mixtures of chemicals that act by mechanisms for which the additivity assumptions is invalid, would

often greatly overestimate their health risks. It can easily be calculated that simultaneous consumption of nutrients at their recommended intake levels would result in an adverse effect level for the mixture when the dose additivity concept is applied.

7.4. Limitations

There are a variety of problems with risk assessment of chemical mixtures in food. In terms of hazard identification the testing of all kinds of (complex) mixtures of chemicals existing in the real world or of all possible combinations of chemicals of a simple (defined) mixture at different dose levels is virtually impossible. Moreover, even if toxicity data on individual compounds were available, one is still faced with the problem of extrapolation of findings obtained at relatively high exposure concentration in laboratory animals to man being exposed to (much) lower concentrations (Henschler et al., 1996). This means that exposure data either on the mixture of choice or on the individual compounds will be necessary for extrapolation. This problem has been recognised as one of the key issues in the assessment of possible health risks form disinfection by-products and contaminants in drinking water (ILSI, 1996; Eisenbrand et al., 2000).

Another important limitation in the hazard characterisation of chemical mixtures is the use of (in)appropriate study designs. The simplest way to study the toxicity of mixtures is to compare the effect of a mixture with the effects of all its constituents at comparable concentrations and duration of exposure at one dose level without testing all possible combinations of chemicals. This strategy has been used to assess the combined toxicity of undefined (drinking water, coke oven emission, cigarette smoke) and defined chemical mixtures (nephrotoxicants, pesticides, carcinogens and fertilisers) (Cassee et al., 1998). Although this approach requires a minimum number of experimental groups ($n+1$, the number of compounds in a mixture plus the mixture itself), it will not be possible to describe the effect of the mixture in terms of synergism or antagonism for every pair of chemicals if there are no dose–effect curves of each of the single compounds. Any time a mixture consists of more than two compounds many two or three factor interactions are likely. If all these factors are carefully thought about in an experimental set-up the number of possible test combinations increases exponentially with increasing numbers of compounds in a mixture. Likewise, the number of experimental groups will also increase with the number of doses of each compound. Full study designs, however, lead to very costly experiments, and even if only two dose levels are used, it is already virtually impossible to perform complete, conventional toxicity tests using 2^n-1 test groups to identify interactions between all chemicals of interest. Both humane and practical reasons force researchers to lower the number of experimental groups. Therefore, a number of statistical designs are available to evaluate as efficiently as possible the effects of mixtures compared to their constituents (Gennings, 1996; Schoen, 1996; Tajima et al., 2002).

7.5. Potential for improvement of the hazard characterisation of chemical mixtures

7.5.1. Hazard index

Despite the necessity to carry out simple test case studies with chemical mixtures, it is obvious that there is a need for generic methods for risk assessment. One approach to approximate the risk posed by exposure to the mixture is the hazard index (HI) as originally put forward in the US EPA mixture guidelines (EPA, 1986). In this approach, the hazard quotients are calculated for individual compounds and the quotients for each compound in the mixture are then added.

It will often be impossible to obtain sufficient and adequate toxicological information on each of the compounds of a mixture to make these calculations. Data on interaction are not included in this approach, and may overestimate the possibility of joint action. For instance, a mixture of nutrients is an excellent example to illustrate that application of the dose addition concept (as a default concept) for assessing the health risks of mixtures of chemicals that act by mechanisms for which the additivity assumption is invalid, would often greatly overestimate their health risks. It can easily be calculated that simultaneous consumption of nutrients at their recommended intake levels would result in an adverse effect level for the mixture when we apply the dose-additivity concept.

A different approach, originally published by Mumtaz and Durking in 1992, takes into account both synergistic and antagonistic interactions in the derivation of the HI. In this approach a weight-of-evidence (WOE) classification is followed to estimate the joint actions (additivity, antagonism and synergism) for binary mixtures of chemicals based on information about the individual compounds. In the WOE method, several weighing factors are used in the final classification such as the mechanistic understanding of the binary interactions, the demonstration of toxicity, and additional uncertainty factors, such as modifiers of interactions, such as route of exposure, in vitro data etc. The better the data set on the individual compounds, the more precise the joint action can be predicted. The HI method can be regarded as a general first assessment of the risk of joint action. The WOE method should be used as a follow-up in those cases where priority mixtures have been established. In order to show its usefulness in future risk assessment, the WOE method has to be validated first with experimental studies as illustrated by Mumtaz et al. (1998).

7.5.2. Comparative potency approaches

7.5.2.1. Toxic Equivalency Factor (TEF) approach. Another strategy to assess the hazard of mixed exposure is the TEF approach, a method which has been applied for certain groups of environmental contaminants. The basis of the concept is that structurally related chemicals may exhibit similar toxicity, and therefore may show joint actions. For example, the group of widespread environmental contaminants polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and dioxin-like PCBs, share a common mechanism of toxic action mediated via interaction with a specific intracellular binding protein, the Ah receptor. The individual congeners differ in their toxic potency and usually occur in foods as a complex mixture. In order to assess the combined effects, each congener has been allocated a TEF relative to the most potent congener, TCDD. The total potency of the combined occurrence of PCDDs, PCDFs and dioxin-like PCBs is calculated as the sum of the concentration of each individual congener multiplied by its specific TEF, expressed as toxic equivalents (TEQs). The summation assumes joint action leading to full dose additivity. However, joint action could also result in partial additivity when a congener with a lower potency (or more importantly, a lower efficacy) displaces one with a higher potency/efficacy. This type of uncertainty and others (non-additive interactions, differences in shape of the dose–response curve, and species responsiveness) have been reviewed extensively and have indicated that the TEF concept is still the most plausible method for the hazard assessment for mixtures of halogenated aromatic hydrocarbons with dioxin-like properties (Van den Berg et al., 1998).

Another example where a TEF-like approach has been applied are compounds with estrogenic or anti-estrogenic activities mediated via interaction with the estrogen receptor. Many different compounds can interact with the estrogen receptor and considerable attention is currently paid to assessing the human health risk of these estrogen-like responses. Besides industrial compounds, food contains endogenous compounds such as bioflavonoids and indol-3-carbinol, which exhibit estrogenic activity. A quantification of dietary levels of industrial and natural estrogens, coupled with their estimated estrogenic potencies in comparison to the most potent estrogen estradiol, allows for an estimate of overall estrogenic activity expressed in estrogen equivalents (Safe, 1995).

For some food components such as food additives, JECFA and other bodies acknowledge that these additives might share common properties, and these are allocated a group ADI. A group ADI may be allocated based on a common mechanism of action of a group of compounds themselves or their common metabolite (see under section 6.3.)

Another strategy to assess the risk on mixed exposure was recently followed by the ILSI Europe Acceptable Daily Intake Task Force, which evaluated the possibility of interactions occurring between the 350 food additives currently approved in the EU (Groten et al., 2000). The strategy chosen was to identify those interactions which theoretically could be of a health concern, based on similar criteria to those used by JECFA to establish group ADI values, but without the expectation of the close structural or metabolic similarities. In total, 65 additives were identified with numerical ADI values. To analyse this list of additives further, the principle was accepted that joint actions and/or interactions would be most likely between compounds that shared a common target organ, and produced similar adverse effects at doses above the NOAEL (see under section 4). Additives which fulfilled the JECFA criterion ''ADI not specified'' were not considered further. Also, effects produced in animals but with no or low relevance to human health were not taken into account. The toxicology data for the prioritised additives were further assessed to determine which of these might share a common effect profile on the target organ (liver and kidney) and also in the light of their possibility to show toxicokinetic interactions. This further analysis revealed that possible joint actions could not be excluded on theoretical grounds for four additives in the liver and three in the kidneys. Finally, exposure considerations are taken into account to assess the health risk of exposure to mixtures of the selected food additives in liver and kidneys.

A similar type of approach has been proposed by the US Pesticides Programs, which is tasked to address the cumulative assessment of risk posed by exposure to multiple pesticides by multiple pathways (including food, drinking water and air). To undertake a cumulative risk assessment for a set of pesticides that have a common mechanism of toxicity OPP follows a procedure in which pesticides are identified that belong to a ''Common Mechanism Group'' (CMG), for which scientifically reliable data demonstrate a common toxic effect by a common mechanism of action. OPP will perform cumulative risk assessment divided in four steps, for example hazard assessment, dose–response evaluation, exposure characterisation and risk characterisation. Steps 1 and 2 will be carried out by using a WOE approach to determine the toxic endpoint that occurs through a common mechanism for the chemicals in a CMG and by establishing a common measure of toxic potency. In the exemplified case of organophosphates, the cumulative risk was established on the basis of dose-additivity (Office of Pesticide Programs, 2000).

All methods (hazard index, equivalency factor, common target organ toxicity) are used in conjunction with information on the exposure data and margins of safety to estimate the health concern of the components in the

mixture. Undoubtedly, the theoretical considerations in hazard characterisation of the mixture should be verified by simple case studies.

7.6. Gap analysis

There is a marked difference between the complexity of a single compound study and a mixture design study. The experimental plan of a mixture design study will mainly depend on the number of compounds of a mixture and on the question of whether it is desirable to assess possible existing interactions between chemicals in a mixture. Despite their potential and current use, mixture design studies need further extensive testing and cross validation.

In principle, to assess interactions, one can make use of mechanistic or empirical models. ''Empirical'' means that only information on doses or concentrations and effects is available in addition to an often empirically selected quantitative dose–response relationship. On the other hand, ''mechanistic'' stands for the fact that additional information on the sequence of reaction steps is available and quantitative parameters are known. For instance, to describe the interaction between chemicals and a target receptor or enzyme, the mechanism of joint action can be described by Michaelis–Menten kinetics. In general, this will result in a phenomenon called ''competitive agonism'' and the ultimate combined effect will be less than expected on the basis of effect addition because the competition for the same receptor (Cassee et al., 1998). In fact this type of interaction can also be considered empirically as a special case of similar joint action (dose addition). A basic problem with mechanistic models is that in most of the combination studies there is a gap in our information that would allow the application of these models. Therefore, currently empirical models play the dominant role.

Also, the hazard characterisation process of chemical mixtures requires a multidisciplinary approach in order to make meaningful progress. This is possible only when experimental toxicologists, epidemiologists, mathematicians, model developers and health assessors collaborate to ensure parallel research in various areas of this field and to justify the selection of those compounds of particular interest for the hazard characterisation of a mixture.

Finally, when assessing the risk of a new food component, regulatory bodies do not undertake a systematic review of all previously approved (but structurally unrelated) compounds, which theoretically may share a common adverse effect with the new component. It is proposed that in future approvals for food chemicals showing target organ toxicity at doses above the NOAEL, one should consider possible interactions with previously approved chemicals, based on the possibility of a common mechanism of toxicity.

8. Micronutrients and nutritional supplements

8.1. Current situation

The increasing availability of fortified foods and nutritional supplements has raised the question as to what levels of intake of micronutrients and supplements might be considered ''safe'' and how the safety might be assessed. In the case of essential nutrients there are clearly two types of risk, those of deficiency and of toxicity associated with excessive intake (Dalton, 1986). Several committees have addressed or are addressing these issues at national and international level, including the Food and Nutrition Board of the US National Academy of Sciences (1998a), and the Scientific Committee on Food of the European Commission. In addition, there is ongoing activity by the International Programme on Chemical Safety (IPCS) addressing the principles and methods for the Assessment of Risk from Essential Trace Elements.

The usual procedures for evaluating the safety of chemicals in food, involving characterisation of the hazard through a detailed experimental toxicological evaluation in animals, determination of the NOAEL or LOAEL and derivation of an ADI by application of appropriate safety or uncertainty factors cannot easily be applied generally to nutrients. Adverse effects arise from intakes that are too low (deficiency) as well as too high (toxicity), and a complete risk analysis has to take account of both types of adverse effect (Mertz, 1995). Furthermore, within a certain range of intake the micronutrient is essential for health but in many cases deficiency would be encountered if uncertainty factors as large as those commonly used for food additives and contaminants were applied to experimentally determined NOAELs based on toxicity. In order to reflect this difference from the conventional ADI, alternative terminology has been applied both in the US (National Academy of Sciences, 1998b) and the European Community. The term ''Tolerable Upper Intake Level (UL)'' has been used, where ''tolerable'' is meant to imply that this level of intake can, with high probability, be tolerated biologically by individuals. The UL was defined by the NAS as ''the highest level of a daily nutrient intake that is likely to pose no risk of adverse health effects to almost all individuals in the general population''. Thus, with nutrients there may need to be two reference intake values, a lower value [e.g. recommended daily allowance (RDA) or reference dose (RfD)] intended to define intakes associated with a low probability of deficiency and the UL defining intakes with a low probability of adverse effects from toxicity. The IPCS has recognised this situation and have used the concept of an ''Acceptable Range of Oral Intake (AROI)'' based on the recognition that there is a homeostatic range of intakes within which the risks of deficiency or toxicity are low

($\leqslant$2.5% of the population at risk of deficiency at the lower bound and of toxicity at the upper end of the range) (WHO, 2002). The incidence of toxicity that would be considered acceptable at the upper level of the AROI, will depend on the nature of the deficiency syndrome, the nature of the toxicity and the degree of separation of the two dose–response relationships.

In the risk assessment of micronutrients and nutritional supplements, attention therefore needs to be given to the range between the RDA or other such recommended intake and the lowest intake leading to adverse effects of overdose since, practically, this is going to limit the safety or uncertainty factors that can be applied to NOAELs or LOAELs. This is shown schematically in Fig. 1(A), in which the factor "X" indicates what multiple of the RDA is represented by the reference dose. The range between deficiency and toxicity may be large, as with ascorbic acid, or narrow, as with zinc and may vary among subsets of the population. In some cases, as indicated in Fig. 1(B), there may be overlap between the RDA for one population subset and the minimum dose resulting in toxicity in another subset. This is the case with retinal, where the RDA for an adult male may be considered to carry an unacceptable risk of teratogenicity in a pregnant female, or zinc where the RfD is less than the RDA for pregnant/lactating females. "Safety factors" as low as 1 have been applied (e.g. for Mg or F) where the adverse effects of excess are judged to be minor or reversible and much larger factors where there is a wide range between

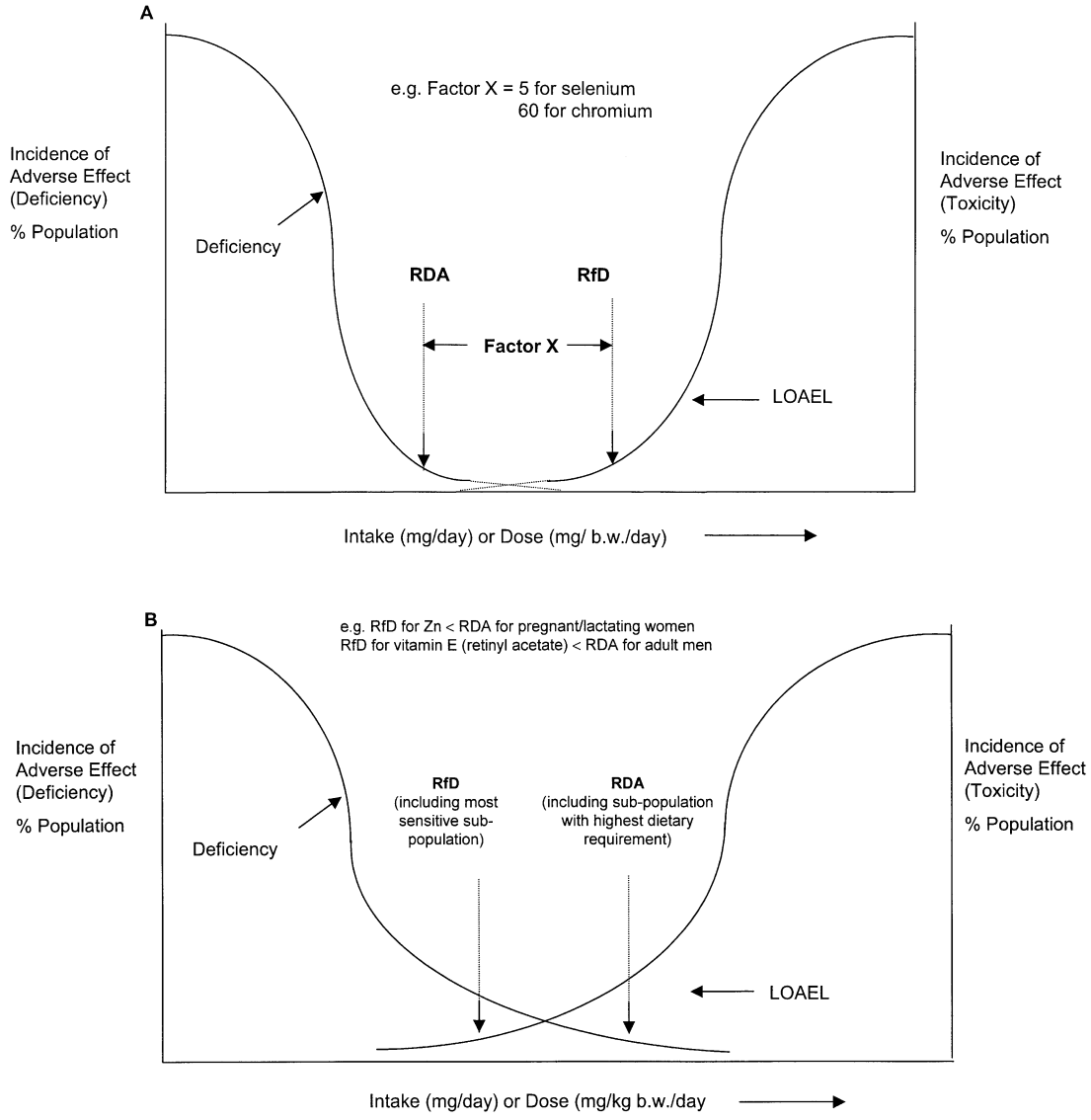

Fig. 1. Relationships of intakes and adverse effects of nutrients. Variable margins between recommended daily allowance (RDA) and reference dose (RfD) for toxicity. LOAEL = lowest observed adverse effect level. Overlap of adverse effects of deficiency in some members of the population with toxicity in others.

deficiency and toxicity, or where the effects of toxicity are severe and irreversible. There is a clear need to develop scientific criteria to underpin these judgements. An additional complication arises from interactions between different nutrients, such as copper and zinc, or vitamins B6, B12 and folate, which may preclude the determination of a unique RDA or NOAEL and require further consideration of how the assessments may be linked. Although the UL is conceptually similar to the ADI, there is an additional complication compared with the derivation of the ADI in that the nutritional requirements of experimental animals may differ both quantitatively and qualitatively from those of humans (the extreme example is the non-essentiality of ascorbic acid in rats and mice). Therefore, human data assume greater significance. There are usually some human data available for nutrients, which, if of adequate quality and extent, would be considered most relevant and given greatest importance in determining ULs, and this may obviate the need to use such large safety factors. However, there are commonly limitations on these data with regard to establishing ULs. There are usually few data from well-controlled studies in humans relating to adverse effects of excessive intake and, where available, these would frequently be restricted to the effects of acute or short-term exposure. Human clinical data relating to the side-effects at high doses used therapeutically, or resulting from abuse of supplements, are often anecdotal and subjective, and overall of limited statistical power. Consequently it may not be possible to determine the chronic LOAEL or NOAEL in humans with any great precision and, ideally, the uncertainty factors applied to derive the ULs should reflect these weaknesses in the database. However, as indicated above, alternative approaches are needed in recognition of the fact that in some cases the margin between individual and population nutritional requirements and the estimate of the tolerable level of intake that is protective of human health may be very small.

An additional complication arises from interactions between different nutrients, which precludes the determination of a unique RDA or NOAEL. This is the situation with some minerals (e.g. Cu and Zn) and vitamins such as vitamin B6, B12 and folate. In fact, in the case of folate, the UL allocated by the US NAS Panel was determined not by the toxicity of folate but by the possibility that high intakes might mask the effects of B12 deficiency by ameliorating the haematological effects without similarly improving the neurological situation. This clearly has a totally different rationale from the ADI approach and if better techniques for diagnosis of B12 deficiency were available, a much higher UL for folate based on direct adverse effects might be indicated.

Variability in nutrient requirements and toxicity may also arise from differences in gender, life stage, physiological conditions and dietary habits. This may be reflected in different RDAs being allocated to different life stages and genders. It is not conventional to make such distinctions in relation to toxicity except on the basis of body weight. This stems from the different approaches adopted by nutritionists and toxicologists. The derivation of the nutritional requirements is based on "risk" or probability curves for deficiency while the toxicological "safety evaluation" is customarily based on a threshold concept. The probabilistic approach used in deriving the AROI as suggested by ILSI RSI/IPCS warrants further consideration and development. As the risks associated with toxicity and deficiency may differ in severity and reversibility, some weighting of the simple probabilistic risk curves may be required so that a lower probability is used for severe, irreversible adverse effects than for minor and reversible outcomes. Additionally, consideration would need to be given to different risks/sensitivities in different population subgroups and at different life stages. As with RDAs, there may be different ULs or AROIs for such subpopulations.

The comparative safety of nutrients has been expressed in terms of the "Safety Index (SI)" defined as the minimum toxic dose (LOAEL) divided by the [RDA, dietary reference value (DRV), etc.] (Hathcock, 1993). An SI of 10 or 1000 would indicate that consumption of 10 or 1000 times the recommended intake provides no margin of safety against the effect seen at the LOAEL. This index needs to be used cautiously in risk management and take into account the nature of the effect seen at the LOAEL and above. An SI of 1 would demand a detailed risk–benefit analysis.

As application of safety factors as low as 10 would result in ADIs below the RDAs in many cases, a different approach has been suggested (Hathcock, 1993) in which Safety Limits (SLs) are derived which approximate to the minimum risk of adverse effects. The SL may be derived using arithmetic or geometric means, for instance $SL = (LOAEL + RDA)/2$ or $SL = (LOAEL \times RDA)^{0.5}$. The choice of which method to adopt would be based on which resultant SLs provide appropriate margins of safety. This principle then seeks to minimise rather than eliminate risk for those problematical nutrients where RDAs for some population subsets may overlap the LOAEL for others. This approach might be subject to some further weighting of the mean to take account of the nature, severity and reversibility of the adverse effects resulting from deficiency versus excess. However, to date, such an approach has not been explicitly adopted by regulatory authorities.

8.2. Issues arising and research needs

As a result of the problems outlined above, a number of issues and related research needs may be identified.

8.2.1. Uncertainties in the hazard characterisation

The most significant sources of uncertainties identified by the US National Research Council (National Academy of Sciences, 1998b) regarding hazard characterisation arise from the following questions:

- What set(s) of hazard characterisation data for a given nutrient should be used for a particular population/subgroup?
- If animal data are used, which endpoints should be considered and what scaling factors should be used (body weight, body surface, dietary intake, etc.)?
- What is the expected variability in bioavailability and in dose response between animals and humans?
- If human data are used, what adverse effects are to be used and what is the expected variability in dose response?
- How should data from subchronic exposure be used to estimate lifetime risk?
- How are data from non-dietary routes to be used?
- How should the threshold dose be estimated for the human population?

Many of these questions are common to hazard characterisation of other chemicals but may raise particular problems for nutrients for the reasons outlined above.

8.2.2. Scaling factors

The ULs determined for most nutrients are expressed in terms of body weight, or at least the recommended ULs for different ages/life stages, have been calculated on this basis. However, the RDAs may be expressed in other terms (e.g. related to calorie intake or unsaturated lipid intake). This raises the issue of which factors should be used, for example, when both nutritional requirements and toxicity may vary with life stage, for example during pregnancy. Both the quantitative and qualitative nature of the hazard may vary with life stage and need to be taken into account.

8.2.3. Identification of human effect levels

As indicated above, the data for determining the human effect levels may be inadequate and consideration may need to be given to techniques for better identifying and quantifying these parameters, for instance by use of appropriate, sensitive biomarkers of exposure/effect.

8.2.4. Nutrient–nutrient interactions

As current knowledge of nutrient/nutrient interactions and the circumstances of individually variable exposure preclude the determination of a unique NOAEL for some nutrients, further information on variability in the population of inter-related nutrients may be helpful in making a risk assessment and establishing appropriate ULs.

9. Novel foods, macronutrients and whole foods

Novel foods, macronutrients and whole foods present a special case because of the quantities that may be ingested by consumers and because nutritional considerations are normally an essential part of safety evaluation. Thus, an integrated approach is needed. As the safety evaluation of macronutrients and whole foods normally combines hazard identification and hazard characterisation, both will be considered in this section. Functional foods are mentioned briefly, in consideration of the fact that several recent safety evaluations of novel foods, macronutrients and whole foods have concerned products designed to produce beneficial physiological properties beyond those of nutritional effects. In general, hazard identification and hazard characterisation of functional foods follows the same approach as that for macronutrients and whole foods. However, functional foods are notable in that a physiological effect in humans is desired. This implies a degree of specificity (benefits without hazards) not sought for traditional food products.

9.1. Novel foods

In the frame of the FOSIE project 'Novel Foods' are listed as a separate category to take into account existing EC legislation. The 'Novel Food Regulation' (European Commission, 1997a), covers all foods and ingredients not previously consumed to a significant degree within the EU. This includes genetically modified organisms (GMO), foods produced via novel processes, as well as plants or animals not previously consumed in the EU. Additives, flavourings and extraction solvents, typically low molecular weight chemicals, are excluded. Safety evaluation of GMOs and GMO-derived foods are not within the scope of this project since they are the subject of another EU project (European Network Projects on Safety Assessment of Genetically Modified Food Crops, ENTRANSFOOD).

While novel foods are listed as a separate category in the regulatory sense, for safety assessment they can fall under any of the other categories described: low molecular weight chemicals, micronutrients, or macronutrients and whole foods, and evaluated accordingly. For example, a traditional plant that is enriched in a specific vitamin or mineral (with no other changes in nutritional value) could be considered as a novel food according to the regulation. The safety assessment for this plant can be performed considering the specific

aspects as described in the previous section on micronutrients, taking into account the source and composition of the plant, as well as its role in the diet in order to estimate intake. Many novel foods, however, will fall into the category of macronutrients or whole foods, which require special considerations for their safety assessment as detailed below.

9.2. Macronutrients and whole foods

9.2.1. Current situation

Traditional foods are safe by selection not by design. Similarly, in the case of many traditional processes, such as the detoxification of cassava by soaking and the action of microbial glycosidases, the safest approach has been arrived at by a process of trial and error over millennia. With the introduction of new foods and processes the need for a more structured approach for safety assessment has become apparent. Because of the similarity in approach, this section focuses on the evaluation of both macronutrients and whole foods.

The current approach to the toxicological evaluation of macronutrients and whole foods is still heavily influenced by the traditional approach that has been developed mainly for food additives and contaminants. Protocols used for the toxicological evaluation of macronutrients and whole foods tend to follow the same international guidelines (e.g. OECD, 1995) as developed for these low molecular weight chemicals. However, hazard identification and characterisation for macronutrients and whole foods is undergoing considerable evolution. There is greater recognition of the need to identify and avoid toxicological effects irrelevant for risk assessment due to high-dose animal feeding and to search for local effects at intestinal level including effects on nutrient absorption and balance. It is apparent that macronutrients and whole foods are quite different from 'low molecular weight compounds', which explains the need for a more adapted approach for safety testing. In particular, the steps of hazard identification and hazard characterisation are not easily separated. Traditionally, both steps rely heavily on in vitro and in vivo animal experimentation. Hazard identification is the first step in order to identify all potential adverse effects that can occur, independent of dose and other factors. Subsequently, hazard characterisation is performed as explained in the introduction to this section. In the case of macronutrients and whole foods there are clear limitations in this traditional approach, since not only toxicological but also nutritional aspects have to be considered. This explains the need for alternative and/or additional approaches, as detailed below.

Table 7 describes some of the differences between low molecular weight chemicals (such as additives and contaminants) and whole foods which illustrate the need for a more adapted approach.

It is increasingly accepted that the term '*wholesomeness*' rather than 'safety' better describes the evaluation of whole foods — this encompasses several considerations, including toxicology, nutrition, microbiology, and environmental effects. Novel foods, macronutrients and whole foods present a special case because of the quantities that might be ingested by consumers and because nutritional considerations are normally an essential part of safety evaluation. Thus an integrated approach is needed. As the safety evaluation of macronutrients and whole foods normally combines hazard identification and characterisation, both will be considered in this section. Functional foods are mentioned briefly, in consideration of the fact that several recent safety evaluations of novel foods, macronutrients and whole foods have concerned products designed to produce beneficial physiological properties beyond those of nutritional effects. In general, hazard identification and characterisation of functional foods follows the same approach as that for macronutrients and whole foods. However, functional foods are notable in that a physiological effect in humans is desired. This implies a degree of specificity (benefits without hazards) not sought for traditional food products.

The heart of the current process of safety assessment of whole foods and macronutrients is based on a comparative principle, whereby the food being assessed is compared with one that has an accepted level of safety (often based on history of safe use). An example of the application of this principle, although a food additive and not a macronutrient, is the comparison of subchronic oral toxicity data of enzymatically depolymerised sodium carboxymethylcellulose (CMC-ENZ) and traditionally used sodium carboxymethylcellulose

Table 7
Differences between low-molecular weight chemicals and whole foods

Additives/contaminants	Food
Simple, chemically defined substance	Complex mixture
Low proportion in the diet (usually less than 1%)	High proportion in diet, high intake (often >10%)
No nutritional impact (with few exceptions)	Nutritional impact possible depending on dose
Specific route of metabolism, often simple to follow	Complex metabolism with interactions
Acute effects obvious	Acute effects difficult to produce (usually absent)

Adapted from JECFA Expert Consultation (2000).

(CMC). CMC has a long history of safe use as a thickening agent and stabiliser in food. Both compounds were tested at doses up to 10% in the diet. Several treatment-related changes were seen in the mid- and high-dose groups for both compounds. As the frequency and severity of changes did not differ between CMC and CMC-ENZ, it was concluded that the two products had a similar toxicological profile (Bär et al., 1995). This conclusion can guide the selection of further targeted toxicity testing based on previous knowledge of traditional CMC.

This principle of '*substantial equivalence*' is not to substitute for safety assessment, but to be an integral and often a core part of the overall safety assessment, guiding toxicological testing in a targeted, case-by-case fashion. Moreover, foods that are found not to be substantially equivalent to a traditional food or food component are not considered 'unsafe' per se. This just indicates that the safety assessment might be more complex and is then based on the unique composition and properties of the novel food. Although the principle of substantial equivalence was originally developed for GMOs, as was the concept of wholesomeness, it is now also applied for the safety assessment of foods from novel sources and processes (European Commission, 1997b; JECFA, 2000).

The concept of substantial equivalence has been further refined, and ILSI Europe has proposed classifications of products based on such equivalence (Jonas et al., 1996): substantially equivalent, partially equivalent or non-equivalent. Besides targeting toxicological testing, the approach avoids unnecessary duplication of animal experiments and exploits the historical data. Importantly, it encourages a comprehensive approach to safety evaluation based on mechanistic insights, nutritional safety and original toxicology where necessary.

9.2.2. Limitations

A fundamental difference between the toxicological evaluation of macronutrients/whole foods and food additives or other compounds occurring at low levels in the diet is the difficulty of using conventional toxicological safety factors in the design of animal feeding studies (Borzelleca, 1992). For whole foods and for ingredients intended for use at high levels of incorporation in the human diet, the feeding of 100-fold or more the concentration in the human diet to experimental animals is usually unachievable. Normally the limit of dietary admixture is approximately 5% (OECD, 1995), above which unintended effects due to nutritional imbalances/deficiencies are likely to occur. For nutrients, dietary incorporation at the expense of a corresponding nutrient is possible at levels as high as 60% (OECD, 1995). However, with increasing levels of dietary incorporation, an increasing number of observed effects are not toxicologically relevant and a consequence of high doses leading to secondary effects, for instance due to nutrient imbalances or metabolic overload. Interpretation of such studies is often difficult and may give rise to erroneous conclusions.

Historically there are several examples that demonstrate this problem. The safety evaluation of modified starches, polyols and other poorly digestible carbohydrates 30 years ago demonstrated the difficulty of interpreting physiological changes on high-dose feeding to rodents. Several such studies reported toxicological effects that appeared to be common to several structurally diverse substances. Such effects included cecal enlargement, nephrocalcinosis and, in long-term studies, adrenal medullary hyperplasia and neoplasia (Roe, 1993). The cecal enlargement seen in the rat has long been accepted to be an adaptive phenomenon without toxicological relevance. It is currently accepted that the nephrocalcinosis seen in such studies may be attributed to disturbances of calcium metabolism (Lynch et al., 1996). The adrenal medullary hyperplasia and phaeochromocytomas seen in long-term studies of xylitol were also attributed to disturbances of calcium homoeostasis (Roe, 1984).

What constitutes a 'high dose' leading to secondary effects that are not due to overt/inherent toxicity of the test compound, however, is highly dependent on the substance to be tested. Therefore, standard approaches for dose selection in rodent feeding studies as developed for low molecular weight chemicals are generally not applicable for macronutrients and whole foods. Generally speaking, biologically and nutritionally inert test materials, such as non-digestible fat substitutes such as olestra, can be fed at higher doses than nutrients with high potency, such as polyunsaturated fatty acids. At high doses, the polyunsaturated fatty acids mandate an appropriate increase in the intake of antioxidant vitamin E to prevent an increase in oxidative stress and subsequent tissue damage in dosed animals. This illustrates the importance of knowledge on the potential nutritional impact of test material before starting feeding studies.

Another important factor in the interpretation of results from animal feeding studies is knowledge of the composition of the test material. As described above, the core starting point for safety assessment of new foods and ingredients is comparison with existing foods with an accepted level of safety. Chemical and physical data for both the test material and the reference food or ingredient need to be available. However, there is often very limited information, for example on natural variability of plant components due to climatic influences or due to plant varieties. Although the need for thorough chemical characterisation of the test material has been emphasised repeatedly by the competent authorities, one of the limitations of the available historical database has been the inadequate characterisation and description of test materials. Most recent studies

address chemical characterisation adequately, with a few exceptions. There may be potential to better utilise the existing data on toxicity of related compounds when conducting hazard identification/characterisation of macronutrients. Unfortunately, much of these data are not published, even though there may be unpublished reports in proprietary form.

9.2.3. Reliability

Although some animal experiments for the assessment of macronutrients have been complicated by the induction of high dose effects, there is no evidence that safety assessments failed to characterise hazards of relevance to humans. For substances such as polyols, starches and fat replacers, the most frequent reported effects in humans concern short-term bowel discomfort, symptoms that can often be predicted from physico-chemical data without recourse to studies in vivo. Many natural dietary constituents produce similar symptoms.

The traditional approach for identification and characterisation of potential hazards from macroingredients, high-dose animal feeding studies, is still applicable where well-defined and relatively inert material is tested. Such material can be tested in rodent feeding studies at levels of dietary incorporation up to 5% or in some cases even higher. However, toxicity studies have to be well designed and adapted to test for potential adverse effects based on previous knowledge of the specific function and nutritional impact of the test material. Examples of such an adapted approach are fat replacers like olestra, or functional ingredients such as the phytosterol esters (PEs).

Olestra is a non-caloric fat replacer that is neither digested nor absorbed. Therefore, the gut is the only organ that is exposed. Consequently toxicological evaluation of Olestra focused on gastrointestinal structure and function and absorption of nutrients from the gut (Thomson et al., 1998).

Phytosterols (plant sterols) are normal dietary constituents. PEs are promoted as nutritionally functional ingredients that can lower blood cholesterol levels by reducing the absorption of cholesterol from the intestine. The cholesterol-lowering function has been demonstrated at levels approximately seven to ten times higher than normal dietary intake of PEs in humans. Foods rich in PEs, for instance fat spreads, have been considered as novel foods in the EU. Besides clinical human studies, PEs have also been tested extensively applying the traditional approach for hazard identification and characterisation, including rodent feeding studies where PEs were incorporated at up 8.1% in the diet (Hepburn et al., 1999). Additional tests for oestrogenic activity have been performed in vitro and in vivo based on the suspected potential of oestrogenicity (Baker et al., 1999). Based on these studies, a safe level of consumption for PEs has been identified.

9.2.4. Potential for future improvements

The limitations of the traditional approach in identifying and characterising potential hazards from new foods and macroingredients has been acknowledged for several years. Several international and national workshops have been held to discuss these limitations and alternative/additional approaches have been proposed (Allgood, 1996; Fix and Allen, 1998; Raiten, 1999). It was agreed unanimously that the safety assessment of new foods and macroingredients has to be done on a case-by-case basis and cannot follow a standardised approach, as is the case for the traditional hazard identification and hazard characterisation of low molecular weight chemicals.

An ILSI Europe Task Force developed guidelines for the safety assessment of novel foods (Jonas et al., 1996). The core of these guidelines is the use of equivalence and similarity evaluation in the safety assessment of food, by the so-called SAFEST approach, which is a guideline on how to apply the concept of substantial equivalence. The SAFEST approach also outlines procedures for safety assessment in the event of partial or non-equivalence. By applying an approach focused on equivalence and similarity, dissimilarities can be identified and safety testing can be directed to the consequences of such differences.

Borzelleca (1996) proposed a model in which the first phase of 'information gathering and assessment' is followed by 'toxicological characterisation' (selected standard toxicological tests), and a third phase of a 'special toxicological test' completes the safety assessment. A similar but more detailed description of an alternative approach to the safety assessment of a macronutrient substitute was given by Munro et al. (1996). A program of mechanistic research aimed at identification of the physical/chemical properties of the test substance, accompanied by metabolic and other toxicokinetic studies, gives guidance for a targeted toxicological testing strategy.

Some general conclusions can be drawn from the foregoing considerations:

- The aim of the safety assessment of macronutrients and whole foods is to provide the same level of safety as for traditional foods. The criterion is therefore not absolute safety, but requires a pragmatic approach based on comparison with traditional foods. This comparative approach, which has been originally defined by the OECD for novel foods, is known as the concept of substantial equivalence. This concept has been further refined by the EU as well as by other scientific groups.
- It is acknowledged that high-dose rodent feeding studies (with the maximum tolerated dose as the top dose) have severe limitations for the

assessment of macronutrients and whole foods, and other approaches are therefore necessary.

- Alternative/additional approaches have been proposed, for example the use of semi-synthetic diets which allows dietary incorporation of a macronutrient at up to 60% (or more), or the application of human-type diets. The latter diets with interchangeable macronutrients are adapted for the nutritional needs of rodents (Huggett et al., 1996), and allow the incorporation of other macronutrients at approximately 20–30%. The test material for animal feeding studies should be in the form as intended for consumption by humans. This ensures that any by-products of processing are included and also avoids the complication of antinutritional or toxic effects of products destroyed by processing.
- Human data are important for the safety assessment of macronutrients and whole foods. More frequent use of human tolerance studies has been advocated as a means of addressing the limitations of animal feeding studies (Borzelleca, 1995; Forbes, 1996). This is of particular importance in the context of hazard characterisation. Controlled testing in humans should be considered as soon as adequate animal data on safety are available. In particular, the nutritional safety of new foods and ingredients has been the subject of attention. Nutritional evaluation is needed:

  i. If the food or ingredient is likely to be consumed in large quantities;
  ii. If the food or ingredient has a specific nutritional function;
  iii. If the foreseeable use of the food or ingredient is likely to cause a nutrient imbalance;
  iv. If a novel process results in a change in nutrient composition not seen in the conventional process;
  v. Where toxicological testing in animals is necessary.

Despite differences in details, most of the proposed alternative/additional approaches for safety assessment of macronutrients and whole foods follow a similar strategy, which can be divided into three phases, namely (1) information gathering and evaluation, (2) comparison with common foods, (3) toxicity testing.

9.2.4.1. Information gathering and evaluation. This comprises:

- chemical, physical and nutritional characterisation
- detailed information on production and processing
- exposure estimation.

All studies should be preceded by a detailed chemical characterisation of the food or macronutrient. The means for conducting such chemical characterisation have advanced considerably in recent years (e.g. with the advent of capillary electrophoresis, the polymerase chain reaction; and matrix-assisted laser desorption time-of-flight mass spectrometry, MALDI-TOF) compared with the methods in use when early novel foods such as single-cell protein were evaluated in the 1970s. As the tools for chemical characterisation evolve in parallel with developments in informatics, new opportunities will arise for characterising and classifying the material under study.

Processing has the potential to increase or decrease the levels of (natural) toxicants present. While it makes sense to conduct toxicological studies on the processed product as intended for consumption, it may be necessary to characterise the effect of processing on the hazards associated with the raw material. Such studies can assist in the development of appropriate process specifications.

9.2.4.2. Comparison with common foods. The concept of compositional equivalence has been introduced to enable better exploitation of existing toxicology data while focusing on the chemical and biological differences that differentiate a new product from conventional counterparts.

9.2.4.3. Toxicity testing. Toxicity testing encompasses:

- specific and targeted toxicological testing
- human clinical trials
- comparative toxicokinetic studies
- mechanistic studies.

Specialised studies on nutritive effects should precede toxicological studies, which might otherwise be difficult to design and interpret due to the possibility of unforeseen nutritional effects. The key here is the degree of specificity in study design compared with conventional toxicological assessment — each macroingredient or whole food being evaluated on a case-by-case basis.

In summary, several proposals for improved testing strategies and overall safety assessment, including hazard identification and hazard characterisation, for macronutrients and whole foods have already been made in order to overcome limitations of traditional safety testing procedures. The common themes in all proposals are extensive characterisation of the new material, and comparison with common foods of accepted safety. The need for early inclusion of human data has been emphasised, as well as the fact that any testing should follow a targeted, case-by-case approach.

All these proposals have to be evaluated in detail and an overall agreement on the necessary steps has to be

developed. A process-based/decision path approach, developing on the decision-tree systems as proposed, for example, by Barlow et al. (2002), would lead to a more systematic assessment of chemical, technological, toxicological and other data in the safety evaluation of macronutrients and whole foods.

9.2.5. Gap analysis and research needs

The general problem of identifying an adaptive vs an adverse response in standard toxicity tests is more complex in the hazard characterisation of macronutrients and whole foods compared to low molecular weight chemicals. In addition to toxicological effects, the nutritional impact of the test material has to be evaluated. This is crucial to permit careful design and interpretation of results from animal feeding studies so as to avoid erroneous conclusions. Furthermore, this requires improved knowledge on nutrient requirements, nutrient interactions and influence on bioavailability of other macro- and micronutrients in rodents and in humans. Proposals have been made for the use of modified rodent diets, or increased use of human data. However, there appears to be little guidance on which approach is appropriate to address specific questions. In addition to the influence on other nutrients, effect(s) on the bioavailability and detoxification of naturally occurring toxicants and contaminants needs consideration. Further research in this area, to establish guidelines on the nutritional boundary conditions for rodent studies, as well as improved collaborations between nutritionists and toxicologists will greatly improve the safety assessments of macronutrients and whole foods.

Another clear difference between macronutrients/whole foods and low molecular weight chemicals is that standard toxicity testing in vivo (for hazard characterisation) cannot encompass an uncertainty factor of at least 100. The need for acceptance of lower uncertainty factors has been acknowledged. However, there is lack of guidance on 'what is acceptable'. In this context, a concept should be developed combining aspects of uncertainty factors and nutritional homeostasis.

Enrichment of foods with a component with a specific biological function is done for beneficial effects on human health or overall well-being. This component can already be a normal part of the diet, albeit at lower doses. These types of foods with functional ingredients require a specific, targeted safety assessment that is guided by prior knowledge of their biological function(s). An example of an area of novel foods that has received little formal attention is the safety assessment of microorganisms intended for addition in viable form to new products. In the case of strains in current use, the history of safe use criterion is very much associated with being able to detect, enumerate and characterise strains to ensure consumers have been ingesting the same organism throughout the claimed exposure period. This is a limitation at present because of the variety of methods available for identification of bacteria and the lack of standardisation/consensus in the area of bacterial taxonomy. Although the principle of substantial equivalence might be applicable in the case of new strains, bacteria are typically more easily characterised by their activity than by composition, as reflected in traditional taxonomic approaches based on metabolic profiles. Thus, metabolic activity both in the food matrix and in the intestine following consumption is an important safety criterion. This means that evaluation should also examine products as intended for human consumption since the matrix and fermentation conditions will affect activity.

Because of the limitations of animal feeding studies, as outlined before, there is an increased need for human data for the hazard identification and characterisation in the safety assessment of macronutrients and whole foods (see van den Brandt et al., 2002). In particular, nutritional testing and tolerance studies are necessary to ensure that the nutritional status of consumers is not jeopardised by substitution of existing foods of known nutritional value (as well as antinutritional effects), with new foods with unknown nutritional effects. The development of suitable biological indicators for nutritional changes in animal studies can be an important factor in improving human studies. Further improvement can be achieved by identifying early indicators of biochemical change ultimately leading to adverse effects (Schilter et al., 1996). Changes in these early indicators are detected at doses lower than those necessary to elicit a toxic response, therefore increasing the sensitivity of toxicological studies. Further research is necessary to validate such approaches in animal studies and test their suitability for human clinical trials.

10. General conclusions and research needs

10.1. General conclusions

The hazard characterisation of low molecular weight chemicals in food and diet is centred on the quantification of the dose–response relationships for observed adverse effects. This generally involves the identification of the NOAEL as a starting point; that is the exposure level at which there are no statistically or biologically significant increases in the frequency or severity of adverse effects. Alternatively, a regression function fitted on the response data may be used to estimate the dose (the benchmark dose) at which adverse effects start to arise. For hazards that are considered not to have thresholds for their mode of action, low-dose extrapolation and other modelling approaches may be applied (see Edler et al., 2002). Hazard characterisation also includes identification of the most sensitive species

and strain, since these are usually used as models for the human situation reflecting the default, worst-case nature of the process. In addition, potential qualitative and quantitative species differences in toxicokinetics and toxicodynamics are identified as inputs to animal to human extrapolations.

The default position in hazard characterisation is that rodents are good models for humans. However, quantitative differences between animals and humans have been described for a number of chemicals, especially relating to their metabolism. Thus, information on such differences is used to modify the default uncertainty factors applied in extrapolation from experimental animals to humans (see Edler et al., 2002). Also, qualitative differences exist between animals and humans in that some chemicals cause species-specific toxicity syndromes. In such instances, the animal data will not be predictive for humans. On the other hand, it should also be recognised that some toxicity syndromes in humans have not been replicated in animal models. A central theme to hazard characterisation is unravelling of the modes of action/the mechanism for the critical effects observed so that their relevance for the human situation can be addressed. As animal experiments are used as models for potential toxic responses in humans, and since the dosages used in the animal experiments are usually much higher than those experienced in human exposures to low molecular weight chemicals in food and diet, hazard characterisation involves both extrapolation from high to low dose and from experimental animals to humans.

Food can be considered as an extremely complex and variable chemical mixture estimated to consist of thousands of chemicals (nutrients, low molecular weight chemicals, plant metabolites). Such chemicals may have simple similar action (dose addition) or simple dissimilar action (independent action), or they may interact resulting in a stronger effect (synergism, potentiation, supra-additivity) or weaker effect (antagonism, inhibition, sub-additivity). One should be cognisant of the possibility for interactions; however, such interactions are rarely expected to be present given that the exposure levels generally are far below their NOAELs.

Hazard characterisation of micronutrients must take account of the fact the adverse effects may arise from intakes that are too low (deficiency) as well as too high (toxicity). Furthermore, within a certain range of intake the micronutrient is essential for health. An additional complication in the hazard characterisation of micronutrients arises from interactions between different nutrients. Variability in nutrient requirements and toxicity may also ensue from differences in gender, life stage, physiological conditions and dietary habits.

The current approach to hazard identification and hazard characterisation of macronutrients and whole foods is still heavily influenced by the traditional approach that has been developed mainly for food additives and contaminants. Owing to limitations in this approach, such as in the design of animal feeding studies and higher possibility of nutritional impact on overall diet, new strategies and concepts have been proposed. The principle of substantial equivalence is applied, whereby the macronutrient and whole food is compared to a food that has an accepted level of safety (often based on history of safe use). Novel foods, as defined by EU regulation, can, from a standpoint of safety evaluation, fall under the categories of either low molecular weight chemicals, micronutrients, or macronutrients and whole foods, and be assessed accordingly. This necessitates that macronutrients and whole foods be evaluated on a case-by-case basis and cannot follow a routine assessment protocol.

10.2. Gaps and research needs

10.2.1. Low molecular weight chemicals

Central to the interpretation and extrapolation of dose response toxicity data is the understanding of the underlying modes of action. Thus, for a number of low molecular weight chemicals where the mode of action is not presently understood, there is an obvious need for mechanistic-type studies. This would especially be needed in situations where the range of human exposures are close the ADI/TDI derived from default assumptions. Also, clarification is necessary of the relevance of the endpoint for humans where there are obvious species differences in response. Mechanistic-type studies should address whether the modes of action for the observed effects may be extrapolated downwards to realistic human exposure levels as well as whether they are relevant for humans.

Further developments in application of non-radiolabelled technology, such as NMR spectroscopy, will presumably increase the understanding of toxicokinetics and thus improve the process of hazard characterisation. However, further validation and experience is necessary and an increasing database will allow for better interpretation of results.

Future application of novel molecular biological methods, including those in genomics and proteomics, will generate a vast amount of data. It will therefore be a major challenge to interpret such data and incorporate them in a hazard identification and characterisation context. This will call for studies in integrated, whole organism models in order to sort out the insignificant responses from critical events. Also, application of the molecular biological methods in conjunction with conventional toxicological studies is warranted in order to develop experience on how data from such methods can be interpreted.

10.2.2. Chemical mixtures

Hazard characterisation of chemicals in food are usually performed on single entities, although food exposures always represent complex mixtures. There is therefore an obvious need for more data on potential interactions of chemicals in food. Despite the potential use of available methods, mixture design studies need further extensive testing and cross-validation.

The hazard characterisation process of chemical mixtures requires a multidisciplinary approach by toxicologists, epidemiologists, mathematicians, model developers and health assessors to justify the selection of those compounds of particular interest for the hazard characterisation of a mixture.

For new food chemicals that show target organ toxicity at doses above the NOAEL, studies are warranted in order to identify any interactions with food chemicals in use, based on the possibility of a common mechanism of toxicity.

10.2.3. Micronutrients and nutritional supplements

There is an obvious need for updating the scientific basis for establishment of lower and upper safe intake levels of micronutrients in food. Particular attention should be given to micronutrients with a narrow safe range of intake. There is also a need to generate more human data on the kinetics and potential adverse health effects of micronutrients and nutritional supplements at different levels of intake, and the variability of these parameters in the population. Increased research on the variability of the kinetics of micronutrients and nutritional supplements related to age, gender, physiological conditions (pregnancy and lactation) and nutritional status (e.g. high fat intake) is warranted.

The bioavailability of the industrially-produced micronutrient may be different from its natural counterpart. Furthermore, synthetic micronutrients may contain isomers that differ from those present in the natural material and thus be metabolised differently, which may result in a different health effect.

Many interactions are described for minerals and trace elements, although mostly in a qualitative way. Both deficient and excessive intakes of minerals and trace elements can cause interactions that have an adverse effect on the absorption and metabolism of other constituents of the diets. Some of the vitamins also have the potential of interacting with other nutrients. Thus, there is a need for research addressing the quantitative interactions between micronutrients, nutritional supplements and other food constituents. It is important that research studies are designed in close collaboration between nutritionists and toxicologists.

10.2.4. Novel foods, macronutrients and whole foods

In the area of safety assessment of novel foods, macronutrients and whole foods, the steps of hazard identification and hazard characterisation are not as separate and sequential as those for low molecular weight chemicals. Safety evaluation is based on a comparison with traditional foods and has to be performed on a case-by-case basis, following a strategy divided in following phases: collection and evaluation of background information, comparison with traditional foods, targeted toxicity testing.

For valid comparison, adequate characterisation and description of the test material is important, and guidelines for (minimal) data requirements would greatly improve the application of the concept of substantial equivalence.

Hazard characterisation of macronutrients and whole foods is complex in that, in addition to identifying potential toxic effects, the nutritional impact of the test material has to be assessed. This implies a need for improved knowledge of nutrient requirements, nutrient interactions and influence on bioavailability of other nutrients in both the test species and in humans. Further research is necessary to establish guidelines on nutritional boundaries for rodent studies. Increased collaboration between nutritionists and toxicologists will greatly improve knowledge on study design and interspecies extrapolations.

The traditional approach for safety assessment based on animal feeding studies is limited in the area of macronutrients and whole foods, and the doses applied cannot encompass an uncertainty factor of at least 100. The need for acceptance of lower uncertainty factors has been acknowledged: however, guidance is needed on what is acceptable. In this context, a concept should be developed combining aspects of uncertainty factors and nutritional homeostasis.

There is clearly an increased need for human data to be included early on in the safety evaluation of macronutrients and whole foods. The development of suitable biological indicators for nutritional changes in animal studies can help to design human studies and so greatly improve their value. Validation for such indicators is required.

Several approaches for guidelines or decision trees on the evaluation of macronutrients and whole foods, as well as foods with a specific nutritional purpose ('functional foods') have been proposed. An overall review of published proposals can serve as a basis to develop overall guidance on improved testing strategies and to develop a common proposal on a process-based/decision tree approach.

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