Committee on Toxicity of Chemicals in Food,
Consumer Products and the Environment

Variability and Uncertainty in Toxicology of Chemicals in Food, Consumer Products and the Environment

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Consumer Products and the Environment

Chair
Professor I Hughes

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Chair of the Working Group
Professor Peter Aggett OBE

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1. Executive Summary

1.1 In 2003, the Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (COT) identified a need to review the approaches that are currently used, or that might be used in future, to identify and compensate for variability and uncertainty in the biological data used in the risk assessment of chemicals in food, consumer products and the environment. The COT set up a new working group, the Working Group on Variability and Uncertainty in Toxicology (VUT), to consider the issue. The membership of the Working Group is listed in Appendix 7. The first meeting of the Working Group was on 14th November 2003 and it subsequently met on five further occasions throughout 2004, 2005 and 2006. The final report of the Working Group was endorsed by the COT on 2nd March 2007.

1.2 The terms of reference of the Working Group were:

- To review the evidence of the bases and range of variability in response to toxic chemicals.
- To consider sources of uncertainty in hazard identification and characterisation.
- To consider the appropriateness of uncertainty factors customarily used to extrapolate toxicological data from animals to humans.
- To consider the appropriateness of uncertainty factors customarily used to allow for variation within the human population, including specific subgroups such as children.
- To consider other methods that might be used in setting acceptable or tolerable intakes for chemicals in food, consumer products and the environment.
- To consider how to express the level of confidence that one can have in the risk assessment.

1.3 In this report the terms “variability” and “uncertainty” are used as defined in the context of chemical risk assessment by the International Programme for Chemical Safety (IPCS, 2004):

- Variability was defined as observable diversity in biological sensitivity or response, and in exposure parameters.
- Uncertainty was defined as imperfect knowledge concerning the present or future state of an organism, system or (sub)population under consideration.

1.4 Risk analysis comprises three stages. These are risk assessment, risk communication and risk management. Risk assessment involves hazard identification, hazard characterisation, exposure assessment, and risk characterisation. Each of these steps is subject to variability and uncertainty in the available information. This report focuses on uncertainty and variability in hazard identification and characterisation, and does not consider those in exposure assessment. Hazard identification and characterisation are the steps in which the complete database on the toxicity of a chemical is evaluated and used to set a health-based guidance value (sometimes termed a reference dose).

1.5 Health-based guidance values are the levels of exposure that are regarded as causing no appreciable harm in exposed people. They are determined during hazard identification and characterisation. These values include the acceptable daily intake (ADI), the tolerable daily intake (TDI) and the acute reference dose (ARfD). The report considers several approaches (e.g. No Observed Adverse Effect Level – NOAEL, and the Bench Mark Dose Level – BMDL) used in establishing health-based guidance values. In particular, it focuses on the sources of uncertainty and variability involved in the process, and the application of uncertainty factors to compensate for these.

1.6 After a general introduction (Chapter 2), this report summarises in Chapter 3 the risk assessment process and describes the general background in which uncertainty and variability in the available data and information need to be considered. Chapters 4, 5, and 6 describe the sources of variability in the different types of biological data available, and sources of uncertainty are considered in Chapter 7. The effect of variability and uncertainty in relation to using human data is described in Chapter 8. Since they are areas of particular public concern in relation to infants and young children, Chapters 9 and 10 respectively explore variability and uncertainty in the context of neurodevelopmental toxicity and endocrine modulation. The adequacy of the existing risk assessment methods to deal with uncertainty and variability is evaluated in Chapter 11. Alternative approaches that may refine risk assessment are addressed in Chapter 12. The Committee's conclusions and recommendations are summarised in Chapter 13.

1.7 The conclusions are set out below, under the respective terms of reference:

To review the evidence of the bases and range of variability in response to toxic chemicals

1.8 Variability in response to chemicals is determined by the fate of the chemical within the body (toxicokinetics) and the toxicity of the chemical and its metabolites (toxicodynamics). Variability in both toxicokinetics and toxicodynamics arises from a combination of factors that are inherent to the individual (or organism), and other factors that relate to the physiology and environment of the individual and which change over time. Inherent characteristics include species, sex and genotype. The modulating factors include age, stage of development and functional maturation of organs and systems, co-exposure to other agents and compounds (e.g. nutrients), lifestyle, and environmental factors, and disease. In principle, variability is measurable, and any lack of knowledge of variability is a source of uncertainty.

1.9 Recent scientific advances have led to greater insight into genetic factors responsible for susceptibility. It is now becoming possible to explain some variability in terms of genetic polymorphisms and post-genomic molecular biology. However, genetic heterogeneity and gene expression are not new forms of variation. They have always existed as part of the underlying differences between individuals, and as such would have been part of the variability that has informed the development of current methods that are applied to deal with variability in risk assessment.

1.10 The range of human variability in response to chemicals cannot be measured directly in all sectors of the population. It is inferred from studies in different animal species, and from knowledge of the differences between humans and animal models in toxicokinetics and toxicodynamics.

1.11 Each source of variability results in a distribution of activity or functionality amongst individuals of a population, leading to either increased or decreased susceptibility to the toxic effect. The overall response to a toxic chemical is determined by the combination of the many different sources of variability. The factors contributing to variability are, unless linked, unlikely to all act in the same direction.

To consider sources of uncertainty in hazard identification and characterisation

1.12 Uncertainty in hazard identification or characterisation relates to incomplete knowledge of the relevance of the results of studies conducted in animals and experimental models, or of human populations, and of possible effects that have not been adequately investigated or recognised.

1.13 The few available direct data relating to human variability are largely derived from studies in young men. Thus, the extent to which these data reflect the susceptibilities of women, older people, the conceptus, or children is uncertain.

1.14 Often there is uncertainty about the association of early exposure with particular health effects later in life. Improved understanding of the pathogenesis of such effects might enable identification of early predictive markers of significant adverse effects that would reduce this uncertainty.

1.15 There is more uncertainty in the hazard identification and characterisation of contaminants and natural constituents in foods because, unlike food additives and pesticides, they are not subject to a formal approval process requiring systematic studies to support safety assessment.

1.16 Another source of uncertainty relates to interpretation of studies giving apparently contradictory results with no obvious explanation. There is a need for an agreed robust mechanism for assessing the results of studies that give contradictory results, and demonstrating clearly, how the hazard characterisation resolves such problems.

To consider the appropriateness of uncertainty factors customarily used to extrapolate toxicological data from animals to humans

1.17 Differences between the animal species used in laboratory experiments and humans derive from anatomical and physiological differences, as well as the variation in genetic factors that occurs within a species. Data from the available research in which compounds have been studied in both animals and man suggest that the default uncertainty factor of 10 allows adequately for interspecies differences.

1.18 The question of special vulnerability of the developing nervous system to neurotoxicity is addressed by current regulatory testing with specific consideration of neurobehavioural and neurodevelopment outcomes. Similarly, data derived from developmental and reproductive toxicity studies in animals can be extrapolated to humans in considering other effects on the fetus and infant. Results suggest that the current approaches and uncertainty factors are adequate. However, it is recommended that this area be kept under review.

To consider the appropriateness of uncertainty factors customarily used to allow for variation within the human population, including subgroups such as children.

1.19 Inter-individual differences in the activity of xenobiotic metabolising enzymes are often characterised in well defined populations of subjects, focusing only on the pathway of interest. Whilst 10-fold or greater differences have been demonstrated between groups, there are frequently no comparable differences in the overall kinetics of the parent chemical, because of compensation by alternative pathways.

1.20 The default uncertainty factor for interindividual variability has been explored empirically on a number of occasions. This has usually been performed with pharmaceutical agents, but these studies can be related to other chemicals, and they suggest that the default uncertainty factor is generally appropriate.

1.21 With a few exceptions, particularly susceptible subgroups cannot be identified by genotype. Some subgroups are potentially vulnerable due to physiological, dietary or environmental factors. With respect to infants and children, it is recognised that the young can be either more susceptible or less susceptible than adults to the toxicity of particular substances. Since more information on newly introduced human pharmaceutical agents will be expected to be derived directly from observations of treated children, it should in future be possible to test the adequacy of current uncertainty factors in protecting young children.

1.22 The possibly increased susceptibility of the elderly and the consequences of a lifetime of exposure are representatively investigated in chronic toxicity studies. Even so, there is a need for better characterisation of the uncertainties related to possible altered susceptibility arising from environmental, physiological and metabolic changes during the course of life and in older life. An additional uncertainty factor for this is probably unnecessary in most cases but should be considered and decided during hazard characterisation on a case-by-case basis.

To consider other methods that might be used in setting acceptable or tolerable intakes for chemicals in food, consumer products and the environment

1.23 The COT uses current internationally-accepted methods in its risk assessments. These make good use of state-of-the-art knowledge and of the methodologies available to take account of variability and sub-group vulnerability in toxicological data. Given the wide range in primary data quality and the frequent occurrence of critical data gaps, it is not possible to propose the use of any single approach to risk assessment, but rather it is necessary to continue with the present flexible use of the assessment methodology best suited to the specific data set available. As a continuing process, the COT will consider improving and refining the methods and approaches it uses.

1.24 *In vitro* studies have important roles to play in hazard characterisation and investigations of toxicological mechanisms. However, there remain uncertainties with regard to the extrapolation of the results of *in vitro* studies to humans. Complete replacement of animal tests in toxicology is not possible at present.

1.25 Application of the default 100-fold uncertainty factor, which allows for 10-fold factors each for inter- and intra-species variation continues to be a reasonable approach, in the absence of better information. In some instances, e.g. if there is good evidence that humans are not more sensitive than animals, application

of the full 100-fold factor is not necessary. Subdivision of the default uncertainty factors to incorporate chemical-specific toxicokinetic and toxicodynamic adjustment factors should be used, whenever data allow. If chemical-specific adjustment factors are used, the adequacy of the remaining default factors should be explicitly considered.

1.26 Statistical and modelling approaches, including physiologically-based pharmaco- or toxicokinetic models, have been used to refine the risk assessment process. Greater use of such methods, when suitable data are available, would support a more systematic approach to risk assessment. Probabilistic models could be used to explore and quantify uncertainty.

1.27 Description of assumptions and uncertainties in the evaluation is important for transparency of the risk assessment:

- Systematic reviews of the relevant toxicology and epidemiology literature are important tools in hazard identification and hazard characterisation and for the presentation of data.
- There should be a description of the criteria for inclusion or exclusion of studies in a review and details of the uncertainties and variabilities in parameters of interest in both the test subjects (e.g. laboratory animals or human cohorts) and the human population of interest (e.g. consumers or exposed workers).
- The choice of critical event used to set guidance values should be justified.
- The validity and robustness of biomarkers of exposure, intake, susceptibility and outcomes should be discussed, along with environmental and lifestyle factors that might impinge on these factors.
- Vulnerable groups of people should be identified

To consider how to express the level of confidence that one can have in the risk assessment

1.28 The degrees of variability and uncertainty at each stage of a particular risk assessment should be clearly described and communicated to those involved in risk management. This should include identification of whether all relevant responses were investigated. Particular attention should be given to stating assumptions and subjective elements in the risk assessment, justification of the choices of uncertainty factors used, and of the selection of the adverse health effects used as the basis for risk assessment. Transparency in these factors aids an informed assessment of uncertainty and enables risk managers to communicate this to stakeholders. Furthermore, such transparency is particularly important in reconciling differences in risk assessments reached by different expert groups.

Recommendations

1.29 There is a need to introduce methods to increase the transparency and reproducibility of hazard identification and characterisation. Several recommendations are made for future areas of research and changes in policy to ensure such transparency and reproducibility.

1.30 Research needs relate to the following areas:

Addressing the best use of existing data:

- Exploration of methods for assessing the quality of the toxicological evidence and the sources of uncertainty and variability.
- Development of a framework for transparent expression of uncertainty in hazard characterisation, such as addressing and identifying critical data gaps.

Vulnerable sub-groups:

- Improved understanding of the relevance to susceptibility of the genetic polymorphisms that have been identified in human populations.
- Evaluating whether there are specific subgroups not protected by the default uncertainty factors, due to genetic, physiological (e.g. early and older life) or environmental sources of variability.
- Developing valid mechanism-based biomarkers of uptake, effect and susceptibility that would help to identify subgroups at risk.
- Better characterisation of hazards to older people to determine whether current uncertainty factors are appropriate.

Mixtures of substances:

- Improve understanding of the combined effects of chemicals occurring in food.

1.31 In relation to policy and practice, it is recommended that:

Hazard characterisation:

- Hazard identification and characterisation should take into account variability and uncertainty, using a systematic approach that will facilitate transparency and confidence.
- Greater use should be made of statistical and modelling approaches, including probabilistic and physiologically-based pharmaco- and toxicokinetic models. Use of such methods, when suitable data are available, would support a more systematic approach to risk assessment allowing for variability within the human population.
- Subdivision of the default uncertainty factors to incorporate chemical-specific toxicokinetic and toxicodynamic adjusted factors should be used, whenever data allow.

Risk communication:

- The development of a framework for transparent expression of uncertainty in hazard characterisation would enable COT and other committees that perform toxicological evaluations to improve communication of the sources of variability and uncertainty in their risk assessments. Particular attention should be given to describing assumptions and subjective elements of the risk assessment, clearly describing where contradictions in information occur and how the resultant uncertainty is resolved.

2 General Introduction

2.1 In 2003, the Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (COT) identified a need to review the approaches that are currently used, or that might in future be used, for identifying and compensating for variability and uncertainty in the biological data utilised in the risk assessment of chemicals in food, consumer products and the environment. The COT set up a new working group, the Working Group on Variability and Uncertainty in Toxicology (VUT), to consider the issue. The Group did not consider issues relating to assessment of human exposure to substances, as their remit covered only variation and uncertainty in toxicology. However, it was recognised that exposure was another major area (along with toxicology) in which variation and uncertainty could affect the outcome of risk assessments. The membership of the Working Group is listed in Appendix 7. The first meeting of the Working Group was on 14th November 2003 and it subsequently met on four further occasions throughout 2004, 2005 and 2006. The final report of the Working Group was endorsed by the COT on 2nd March 2007.

2.2 Biological data, including those used for risk assessment, are subject to variability and uncertainty. The Working Group adopted the definitions the terms "variability" and "uncertainty" that have been used in the context of chemical risk assessment by the International Programme for Chemical Safety (IPCS, 2004):

- Variability is defined as observable diversity in biological sensitivity or response, and in exposure parameters. Variability is due to inherent biological differences between species, strains, sub-strains and individuals, which cannot be reduced. Variability in response to toxic chemicals is determined by the fate of the chemical within the body (toxicokinetics) and the toxicity of the chemical and its metabolites (toxicodynamics). Variability in both toxicokinetics and toxicodynamics relates to a combination of factors that are inherent to the organism, and other factors relating to the physiology and environment of the individual, which change over time. The inherent characteristics include species, sex and genotype. The modulating factors include the physiology of the individual (e.g. age, stage of development, disease or nutritional deficiency, environment and lifestyle factors) and other chemical exposures originating from diet and lifestyle. In principle, variability is measurable, and lack of knowledge of variability is a source of uncertainty (i.e. uncertainty about the variability).

- Uncertainty is defined as imperfect knowledge concerning the present or future state of an organism, system or (sub) population under consideration. Uncertainty refers to lack of knowledge, which can often be reduced by undertaking appropriate studies or by increasing the sophistication or power of studies.

2.3 Recent scientific advances are leading to increased understanding of sources of biological variability. Furthermore, some sectors of society have become increasingly concerned about possible health effects of chemicals, and they doubt whether public health is adequately protected by current health based guidance values and safety assessments. The COT therefore established a working group (VUT) to revisit the approaches for dealing with variability and uncertainty in the biological data used in risk assessment of chemicals in food, to establish whether these methods are still appropriate.

2.4 The principles described in this report may be applied to other chemical risk assessments, such as for occupational exposure. However, there are differences in exposure, opportunities for control of exposure and in population variability that are beyond the scope of the current review.

2.5 The terms of reference of the working group were:

- To review the evidence of the bases and range of variability in response to toxic chemicals.
- To consider sources of uncertainty in hazard identification and characterisation.
- To consider the appropriateness of uncertainty factors customarily used to extrapolate toxicological data from animals to humans.
- To consider the appropriateness of uncertainty factors customarily used to allow for variation within the human population, including specific subgroups such as children.
- To consider other methods that might be used in setting acceptable or tolerable intakes for chemicals in food, consumer products and the environment.
- To consider how to express the level of confidence that one can have in the risk assessment.

3 Introduction to Risk Assessment

3.1 "Risk assessment" is part of the process of risk analysis. Other, subsequent, components of the process are risk communication, and risk management. The Society for Risk Analysis defines "risk analysis" as: *a detailed examination, including risk assessment, risk evaluation and risk management alternatives, performed to understand the nature of unwanted, negative consequences to human life, health, property or the environment; an analytical process to provide information regarding undesirable events; the process of quantification of the probabilities and expected consequences of identified risks* (SRA, 2006).

3.2 A paradigm for risk assessment of chemicals for effects on humans has evolved since the 1950s and is widely accepted both nationally and internationally. Its primary purpose is the protection of human health. Risk assessment is a scientific process comprising four stages. These are hazard identification, hazard characterisation, exposure assessment and risk characterisation. Exposure assessment, which itself is subject to significant uncertainty and variability, and risk characterisations were beyond the scope of the COT Working Group remit as were risk communication and risk management.

Hazard Identification

3.3 A hazard is "the set of inherent properties of a substance or mixture of substances that makes it capable of causing adverse effects to humans, other organisms or the environment". An adverse effect is "a change in morphology, physiology, growth, development or lifespan of an organism which results in impairment of functional capacity or impairment of capacity to compensate for additional stress saw increase in susceptibility to the harmful effects of other environmental influences. Decisions on whether or not some types of effect are adverse or adaptive are likely to require expert judgment"

3.4 A risk is "the possibility that a harmful event (death, injury or loss) arising from exposure to a chemical or physical agent may occur under specific conditions." The most important element is the recognition of the potential adverse effects on human health relevant to exposure to the chemical concerned.

3.5 Hazard identification is "the identification of the type and nature of adverse effects that an agent has as inherent capacity to cause in an organism, system or (sub)population." It needs a full appraisal of available information to identify toxic effects that the chemical, or its metabolites, may have at tissue and cellular function levels. Both human and animal model data are used but information is obtained predominantly from animal studies. Some of these are systematically acquired through specific studies (see below) and this applies particularly to regulated compounds, including for example chemicals that are proposed as food additives. Other data may be acquired more opportunistically, such as from case studies, and incident reports. This is more often the case for contaminants and pollutants. As a principle, wherever possible, data from human experimental or observational and epidemiological studies are preferred. Studies *in vitro* and analysis of structure-activity relationships (SARs) may also be useful.

3.6 The endpoints assessed in regulatory animal studies that involve regulated compounds are those that were available when regulatory toxicology was developed in the 1950s and which have been subsequently refined in the light of emerging knowledge. In general, the databases are likely to be less complete for compounds that were assessed and introduced to the market many years ago, when testing requirements were less stringent, than they are for those subjected to more recent toxicological investigations.

3.7 A comprehensive set of data from such studies would now include: toxicokinetic studies in experimental animals and man; acute, short-term and long-term repeat dose toxicity studies; studies of genotoxicity *in vitro* and *in vivo*; carcinogenicity studies, reproductive toxicity studies (fertility, developmental effects and peri-natal toxicity), and investigations of neurotoxicity and immunotoxicity. Additionally, and usually not relevant to exposure via food, skin irritancy and sensitisation are usually evaluated for regulated compounds e.g. pesticides. In some instances, it may be considered that some studies can be omitted, but this is not usual.

3.8 The studies commonly carried out and the endpoints assessed for chemicals such as pesticides and food additives, which are subject to premarketing authorisation systems, are listed in Table A1 of Appendix 1. It should be emphasised that for food contaminants, many of the studies in Table A1, as well as studies of absorption, distribution, metabolism and excretion, will not be available.

3.9 Neurotoxicological studies that have been added to the standard data package include both single dosing and repeat dosing (13 weeks) which are undertaken with a battery of functional observational neurobehavioural tests. At examination post mortem brain weight is recorded and the brain and spinal cord are subjected to detailed histopathological examination. Peripheral (e.g. sciatic, sural and tibial) nerves are also examined. A recent introduction has been developmental neurotoxicity testing for pesticides (see Chapter 9).

3.10 The molecular techniques of toxicogenomics, proteomics and metabolomics are being used increasingly in toxicological research, but have not yet been incorporated into guidelines for regulatory toxicology studies. The COT and its sister Committees on Mutagenicity (COM) and Carcinogenicity (COC) regularly review developments in this area. Their most recent opinion confirmed that these techniques are potentially valuable adjuncts to conventional toxicology studies, but further research and validation is required before they can be considered for routine use in regulatory toxicological assessments (COT/COM/COC, 2004). Some of the research needs listed in Chapter 13 of this report do, however, involve aspects of risk assessment in which markers derived from such techniques might be useful.

Hazard Characterisation

3.11 Hazard characterisation is the quantitative consideration by dose-response evaluation for the population studied of the nature, relevance and mode of action of adverse effects produced by a chemical. It includes investigation of the ways in which a chemical is dealt with in the body (absorption, distribution, metabolism and elimination, collectively referred to as toxicokinetics) and the toxic effects of the chemical on the body (referred to as toxicodynamics). As was mentioned above, information is obtained predominantly from animal studies, but also, where possible, from human experimental and/or epidemiological studies. Toxicokinetic and metabolic studies may enable the matching of toxicological exposure to adverse effects, as well as help to establish whether metabolites or the parent compounds are responsible for effects. Furthermore, such studies may aid in understanding species and interindividual differences in toxicity. This stage should result in the identification of a health-based guidance value and an account of the uncertainties inherent in the derivation of that value.

3.12 Hazard characterisation may include evaluation of mechanisms of action and of species differences in response, where such data are available. Provided that it can reasonably be assumed that the toxicity is subject to a threshold, i.e. below a certain level of exposure, the substance is thought not to exert adverse effects, this threshold exposure may be used to derive a health-based guidance value. In very general terms health-based guidance values are obtained by dividing a level of exposure found not to cause adverse effects (the no observed adverse effect level (NOAEL)) by an uncertainty factor (sometimes known as a safety factor) that represents the uncertainties in the accuracy of NOAEL and extrapolating it to sensitive subgroups of the population (see below under the heading "Health-based guidance values").

3.13 An important assumption where animal data form the basis of the assessment, is that humans are more sensitive than the most sensitive test species, and (in the absence of evidence to the contrary) that the mechanism of action is relevant to humans. It is also assumed that not all individuals react in the same way to a chemical, and safety guidelines (or advice) produced as a result of a risk assessment attempt to protect the most sensitive individuals within a population by taking into account the potential for inter-individual variability.

Health-based guidance values

3.14 The acceptable daily intake (ADI), tolerable daily intake (TDI) and the acute reference dose (ARfD) are often collectively termed health-based guidance values or reference doses. Customarily, the term "acceptable" is applied for chemicals permitted to be used in food production, and "tolerable" is applied for those unavoidably present, such as contaminants. The ADI or TDI is defined as the daily intake of a substance in food that, in the light of present knowledge, can be consumed every day for a lifetime with no appreciable harmful effects. With some substances, notably pesticides, the ARfD, is also calculated, often from shorter-term studies than those that would support the ADI. The ARfD is defined as the amount of a substance in food that, in the light of present knowledge, can be consumed in the course of a day or at a single meal with no adverse effects. At present most ARfDs for pesticides are conservative since data from subchronic studies (e.g. 90-day studies) may have to be used if specifically designed acute studies have not been performed: exceptions to this are pesticides reviewed or placed on the market very recently.

3.15 The NOAEL based approach is that most frequently used to establish a health based guidance value. This is particularly true with assessments of chemicals subject to pre-marketing authorisation regulatory processes. However, there is no obligation to use this method and other methods for hazard characterisation are discussed in Chapter 12. The NOAEL is generally sought in animal studies, although, if data were available, a NOAEL based on human data would be preferred. However, such data are rarely available for ingested chemicals except for epidemiological studies on some food contaminants and experimental human volunteer studies on some pesticides and some substances that have been developed as human medicines. Furthermore, some countries will not for ethical reasons use human experimental data in hazard characterisation (see Chapter 8). It is sometimes stated that the most relevant NOAEL should be derived from a study in the species most closely resembling humans, but in practice the available data are from a limited number of species (mainly rodent, and sometimes dog or rabbit depending on the regulatory requirements for different products and for different toxicological tests).

The No Observed Adverse Effect Level (NOAEL)

3.16 In any given study, the NOAEL is the highest tested dose at which no adverse effect is observed. The concept of the NOAEL uses a single point (one of the experimental doses tested) as an estimate of threshold. Formally, the rest of the dose response relationship is ignored, although the experienced toxicologist will look at other dose groups to see whether there is a credible dose-response relationship (see also Chapter 7). The NOAEL is dependent on appropriate measurement of likely adverse outcomes. Typically a compound or its metabolites may exert a range of different adverse effects. Usually, the one occurring at the lowest dose or exposure is selected as the critical effect, and its corresponding NOAEL is used to derive the guidance value. It is possible, however, to select from the range of adverse effects that a compound has, another critical effect for risk assessment that is used to set the guidance value. Since each effect may have its own particular NOAEL and associated uncertainty, it is feasible to derive more secure and perhaps lower guidance values from effects other than that which occurs at the lowest level of exposure.

3.17 For nutrients such as vitamins and minerals, the health-based guidance value that is indicated by applying the uncertainty factor might be less than the reference nutrient intake. Furthermore, variability in the response of individuals has the potential to result in a situation in which a given level of exposure could be essential for some individuals but toxic for others.

The Lowest Observed Adverse Effect Level (LOAEL)

3.18 Sometimes it is not possible to identify a NOAEL from the available experimental or epidemiological information. When this happens, it might be possible to use the LOAEL to derive the health-based guidance value. The LOAEL is the lowest administered dose of a substance found to cause an adverse effect. The best circumstances in which to use this approach is when the size of the adverse effect at the LOAEL is small or the shape of the dose response curve implies that the NOAEL would be found at a dose that was slightly less than the LOAEL, or both of these characteristics. It is sometimes possible to combine studies, or to look at preliminary range-finding studies, to obtain some idea of where the NOAEL is likely to be. Like the NOAEL, the LOAEL is dependent on appropriate identification and measurement of likely adverse effects.

The Uncertainty Factor

3.19 The use of uncertainty factors in chemical risk assessment by UK Government departments and agencies recently has been reviewed by the Interdepartmental Group on the Health Risks from Chemicals – IGHRC (IEH, 2003). For over 40 years, an uncertainty factor of 100 has usually been used for extrapolating experimental data from the NOAEL for the critical effect in animal studies to produce health-based guidance values/reference doses (Lehman and Fitzhugh, 1954; Dourson and Stara, 1983; Dourson *et al.*, 1996) for substances such as food additives, pesticides, veterinary medicines and food contaminants. The use of an uncertainty factor is not practical for all substances. For instance, with natural toxicants it might not be possible to reduce exposure to the level suggested by applying the uncertainty factor. Similarly, for nutrients such as vitamins and minerals the health based guidance value that is indicated by applying the uncertainty factor might be less than the reference nutrient Intake. Furthermore, variability in the

responses of individuals has the potential to result in a situation in which a given level of exposure could be essential for some individuals but toxic for others.

3.20 The 100-fold value is considered to comprise a 10-fold factor for interspecies extrapolation and a 10-fold factor to cover human variability (WHO, 1987). It is noteworthy that this approach to uncertainty is also applied to veterinary pharmaceutical agents to extrapolate results from laboratory animals to the animal species to be treated. Thus the species differences and variability relate to the species of interest, which may be farm animals, pets, etc.

3.21 It has been suggested that the 10-fold factor for the variability of the human population may be insufficient to cover certain subgroups in the population, for example children, the elderly and those with certain genetic polymorphisms (see below), having certain illnesses or taking certain medicines. The recognition that the single value of 100 provides no mechanism for exploiting partial knowledge on the magnitude of inter-species and interindividual differences led to the concept of subdividing each of the two 10-fold uncertainty factors into toxicokinetic and toxicodynamic components (Renwick, 1993). This allows relevant chemical-specific data to be introduced into risk assessment (see Chapter 12).

3.22 The concepts discussed above illustrate the need to bear in mind that variations in the toxicokinetics and/or toxicodynamics of a compound may result from differences in the age, diet, physical well-being and genetic make up of individuals. In addition the environment may contribute to differences. Moreover many differences in the toxicokinetics of compounds and/or toxicodynamics also exist between species, illustrating that care must be taken when extrapolating toxicological data from model animal species to humans. These factors must be borne in mind when selecting uncertainty factors as part of a risk assessment. Attempts have been made to quantify what proportion of the human population uncertainty factors would cover and these are discussed in Chapter 11 (paragraph 11.8).

3.23 The default 100-fold uncertainty factor may be increased if there are additional uncertainties in the data, for example if the toxicological data that are available for evaluation are poor and in some other circumstances (see Table 3.1). Inevitably this will involve expert appraisal and judgement. Compound uncertainty factors may be used: thus the JMPR ADI for carbaryl was based upon the LOAEL for vascular tumours in mice, which were considered to arise by a non-genotoxic mechanism, with a 2000-fold uncertainty factor (FAO/WHO, 2002). This large uncertainty factor comprised of the usual 10-fold sub-factors for interspecies and intraspecies variation and an extra sub-factor of 20 to account for uncertainty about the NOAEL. The meeting was mindful that these were rare tumours and that no NOAEL had been established.

Table 3.1 Typical uncertainty factors currently used in regulatory toxicology.

Uncertainty factors	Use	Value
Intra-species (interhuman)	When extrapolating long term studies to provide acceptable daily intakes or short term studies to produce acute reference doses in the same species	10
Inter-species	When extrapolating, from one species to another, long term studies to provide acceptable daily intakes or short term studies to produce acute reference doses	10
Subchronic to chronic	Where no adequate chronic study is available	Up to 10
LOAEL to NOAEL	If the critical effect in the critical study is a LOAEL	Up to 10 (often 3 based on dose spacing)
Incomplete data base	Where the standard data package is not complete	Up to 10
Steep dose-response curve	Where the dose-response curve for a compound is steep, a small error in extrapolation would have dramatic consequences	See text

3.24 When the critical toxicological endpoint has been particularly serious, an extra uncertainty factor has on some occasions been used (Renwick, 1995). An extra factor might be required in certain situations that might include non-genotoxic carcinogenicity (especially for rare tumours where the pathogenic mechanism is unknown), teratogenicity, fetotoxicity and developmental neurotoxicity[1] in the absence of observed maternal adverse effects. The extra uncertainty factor might give additional assurance that people exposed at doses less than the health-based guidance value will not subsequently suffer serious irreversible adverse effects. The main problem in using extra uncertainty factors is the subjectivity of their application and the values involved. The process itself is a source of uncertainty. For example, Renwick (1995) has drawn attention to the illogicality of responding to a serious adverse effect which occurs at a higher exposure level by increasing the uncertainty factor used to set a guidance value from a NOAEL for another effect that had been identified as the critical effect. An example of this was the assessment of the pesticide triazophos, where the 1991 JMPR temporary ADI was based on plasma and erythrocyte cholinesterase inhibition and a safety factor of 500, because of "uncertainty regarding the potential of triazophos to cause delayed neurotoxicity", despite there being no evidence of such a problem in the relevant dog study (FAO/WHO, 1991).

3.25 The carcinogenicity of compounds that are not genotoxic is an issue for which there has previously been a lack of consistency in deciding on the need for an additional uncertainty factor. There is a developing consensus that knowledge of the mechanism of non-genotoxic carcinogenesis should be used in the risk assessment. If another form of toxicity is seen at doses below those causing cancer, then the health-based guidance value should be set using the NOAEL for that toxicity and the default uncertainty factor, and this would also be protective against cancer. Recent evaluations of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) are an example of this (COT, 2001).

3.26 An extra uncertainty factor of up to 10 has been proposed where no adequate chronic study is available. This is unlikely to be the case with substances subject to pre-marketing approval because approval would

1 There is comparatively little experience of developmental neurotoxicity tests at present and consequently consensus has yet to emerge on the appropriate regulatory response to results of these tests.

not be given until the data were available, but may well be the case with food contaminants. However in the case of drugs, Lumley and Walker (1986) suggested that studies of greater than 6 months did not add significantly to knowledge of the safety of the compounds, with the exception of carcinogenicity endpoints. Other workers have suggested smaller uncertainty factors than 10: Kalberlah *et al.* (2002) suggested factors based on decreases in effect concentrations of 3.2 (for extrapolation from subacute to subchronic exposure), 2.7 (subchronic ➛ chronic) and 6.6 (subacute ➛ chronic), after analysing 46 technical reports of subacute, subchronic and chronic studies of respiratory toxicants that were performed as part of the United States National Toxicology Program (NTP).

3.27 More obvious is the need for an extra factor (typically up to 10), where the critical effect in the critical study is a LOAEL rather than a NOAEL (see paragraph 3.15). The magnitude of this factor would logically depend upon how near, in the toxicologist's judgement, the LOAEL is to the undetermined NOAEL. It would be inappropriate to use a LOAEL to set a health-based guidance value/reference dose if the undetermined NOAEL were to be judged to be more than ten times less than the LOAEL. Also, if the effects seen at the LOAEL are considered to be indicators of mild damage (e.g. biochemical changes), a smaller additional uncertainty factor might be indicated compared to a situation where gross pathological damage is seen at the LOAEL.

3.28 Where the standard data package for a regulated chemical is incomplete, an additional uncertainty factor may be required. The reason for this is that an incomplete data package, or one dependent on studies where endpoints that might prove critical were not measured, might produce a higher critical NOAEL than would be provided by a more complete data package. A particular problem with food contaminants is that the data on reproductive toxicity are often poor.

3.29 A steep dose-response curve (i.e. when a small increase in exposure is associated with a large response in the adverse effect) has occasionally been used to justify an extra uncertainty factor (reviewed in Renwick, 1995). Where the dose-response curve for a compound is steep, there could be more serious consequences for those exposed to intakes above the health-based guidance value: a corollary of this is that a steep dose-response curve would give greater confidence in the NOAEL (Renwick and Walker, 1993).

Exposure Assessment

3.30 Intake assessment comprises the measurement or estimation of exposure to a chemical by any route for the population or subgroups thereof (e.g. toddlers, children, adults, ethnic groups). It includes consideration of the pattern, frequency and duration of exposure and is subject to considerable uncertainty and variability. The overall risk assessment is affected by uncertainty and variability in both the exposure assessment and in the toxicological data. This document deals only with variability and uncertainty in toxicology as this was the remit given to the VUT, but it is recognised that variability and uncertainty in exposure assessment is also a major influence on the risk assessment process and this has been addressed by EFSA (EFSA, 2006a).

Risk Characterisation

3.31 Risk characterisation is the combined consideration of hazard identification, hazard characterisation and exposure assessment to predict whether effects in humans are likely and the probable nature and severity of such effects. If data permit, it may include identification of the proportion of the population affected and the existence of any vulnerable sub-populations. Risk characterisation is the synthesis of exposure assessment and hazard characterisation, and therefore incorporates two sources of uncertainty and variability.

Use of Risk Assessment

3.32 Risk assessment may be used to help suggest appropriate risk management measures, to inform policy development, to advise politicians or communicate with the public. In all instances the recipient of the risk assessment will need to be made aware of the variability and uncertainty in the risk assessment. Where chemicals are subject to pre-marketing authorisation, risk assessment is usually mandated as part of the regulatory process. Pesticides, biocides, human and veterinary medicines, food additives and animal feed additives are examples of regulated chemicals. A major difference exists between such chemicals that are authorised for direct or indirect food use and have a sponsor, and those that do not e.g. food contaminants. In the case of the former, authorities can require the sponsor to fill gaps in the database prior to approval, whereas with the latter, there is no obvious person or organisation responsible for filling any gaps in the database. Nevertheless, risk assessment is required to support the establishment of regulatory limits for contaminants in food, and to underpin advice on food consumption. There is a considerable degree of agreement on what data are required for toxicological evaluation of chemicals. Large gaps in the database might necessitate a precautionary risk assessment.

Chemicals in food

3.33 Committees such as the Scientific Committee on Food (SCF), the International Co-operation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH), the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Joint FAO/WHO Expert Committee on Pesticide Residues (JMPR) have produced guidance for the evaluation of food additives, contaminants and residues of veterinary medicines or pesticides (EC, 2001; VICH, 2005; WHO, 1987; 1990; 1994). These specify the range of toxicological tests that are generally required, as listed in Table A1 of Appendix 1. However, no fixed programme is defined and the studies required will depend on the chemical nature of the additive, its (proposed) uses and levels in food. A full set of toxicological studies may not be considered necessary if substances are metabolised to normal constituents of the diet or body.

3.34 In addition to laboratory tests, it may be possible to use human data derived from epidemiological, occupational or volunteer studies. Human studies should not be used to establish general safety of a food additive and should only be proposed if there are adequate data from animal and other studies to demonstrate the likely safety of the proposed level of exposure. They may be used to investigate human toxicokinetics, in support of establishing the relevance of animal data. They may also be used for investigating tolerance to a substance, for example by investigating symptoms (e.g. headaches, gastrointestinal discomfort) that cannot be studied in animals. There may be specific requirements

depending on the population that is likely to be most exposed. For example, evaluation of an intense sweetener is likely to include studies of tolerance in diabetic subjects, who may be expected to have higher than average dietary exposure.

3.35 There are reduced requirements for the testing of certain classes of substances, such as flavouring agents, for which dietary exposure by humans is often very low. The procedure followed by JECFA for non-genotoxic flavouring agents (WHO, 1997) is similar to the threshold of toxicological concern (TTC) approach described at paragraph 3.42.

3.36 Standard testing protocols do not adequately model feeding in the neonatal phase with infant formulae and the ADI is not generally considered applicable to infants under 12 weeks of age. If an additive is proposed for use in infant formula, a special dosing regimen might be required to fully establish its safety. This could involve animal studies using oral administration direct to the offspring, from birth through to weaning.

Tests on specific groups of pesticides

3.37 With specific groups of compounds certain additional endpoints are measured in the standard studies, notably for organophosphorus (OP) and carbamate anticholinesterase pesticides, acetylcholinesterase activity in erythrocytes and/or brain and butyrylcholinesterase activity in plasma or serum are measured. Also, with certain groups of compounds, extra studies may be routinely carried out. An example is the test in hens for the potential of OPs to cause OP-induced delayed polyneuropathy (OPIDP). In this test, hens are dosed singly and repeatedly *inter alia* at doses causing severe acetylcholinesterase depression, while being treated with atropine and a pyridinium oxime cholinesterase reactivator to allow them to survive such high and otherwise lethal doses. The endpoints measured are clinical (eg. any ataxia), histopathological (characteristic changes of OPIDP in peripheral nerves and spinal cord) and biochemical (inhibition of neuropathy target esterase). OPIDP is discussed further in the section below entitled "Risk assessment in circumstances where it is not possible to set a health-based guidance value/reference dose".

Risk assessment in the UK

3.38 There is a considerable degree of agreement on risk assessment strategies between the UK Agencies and Government Departments that undertake risk assessment of the health effects of exposure to chemicals, although there are some differences in detail. This consistency is facilitated by the work of the Interdepartmental Group on Health Risks from Chemicals (IGHRC) and the documents it publishes. These include a review of the use of uncertainty factors in chemical risk assessment by UK Government and agencies (IEH, 2003). This agreement extends to study design where internationally-agreed protocols are widely adopted (IEH, 1999a & 1999b).

International harmonisation

3.39 Internationally, there has been harmonisation of data requirements for food additives, veterinary medicines and pesticides, under the auspices of the Codex Alimentarius Commission. There has been harmonisation between the EU, USA and Japan of data requirements for drugs under the International

Co-operations on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) and International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH). There has also been international harmonisation of study guidelines at the Organization for Economic Cooperation and Development (OECD).

Risk Assessment in Circumstances where it is not possible to set a Health-Based Guidance Value

Events elicited during hazard identification where an As-Low-As-Reasonably Practicable (ALARP) approach may be adopted.

3.40 For substances that are genotoxic and carcinogenic, it is not considered possible to identify a threshold, because there could be some risk, albeit small, at any level of exposure (COC, 2004). In these instances the Committee on Carcinogenicity of Chemicals in Food, Consumer Products and the Environment (COC) advises that exposure should be as low as reasonably practicable (ALARP). This means that genotoxic carcinogens are not usually permitted as substances subject to premarketing authorisation processes, particularly those used in food production. Establishment of ALARP for genotoxins that are unavoidable contaminants of the diet (e.g. aflatoxins, polycyclic aromatic hydrocarbons, acrylamide) is a risk management issue, and setting maximum permitted levels takes into account what can reasonably be achieved (and monitored) in different types of food. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the European Food Safety Authority (EFSA) have recently proposed a Margin of Exposure (MOE) approach to help prioritise risk and support risk management decisions in such situations (WHO, 2005; EFSA, 2005a). In some other countries, notably the USA, different approaches such as quantitative risk assessment based on tumour incidences in animals and the estimation of a "virtually safe dose" are used.

3.41 Likewise, with OP insecticides (in pesticides and veterinary medicines), the propensity to cause OPIDP is usually considered a reason for refusing approval for marketing them. OPIDP has a clear dose-effect threshold, but the reason for not approving marketing of OPs that cause OPIDP is the irreversible and untreatable nature of the neuropathy. In the case of the delayed polyneuropathy produced by some OPs such as tri-orthocresylphosphate (in the past a human food contaminant), there is good evidence for differential sensitivity across species. Rats and mice, although not completely resistant to morphological signs of damage, did not develop motor signs of a neuropathy. Some species, such as ferret, cat and buffalo, proved as susceptible as humans, but are not generally used for routine toxicological testing. The customary species for toxicological testing is the hen (Johnson, 1975) since hens show clear motor signs at similar doses to humans and are relatively easy to use in routine testing. The relationship between the primary biochemical action associated with induction of the delayed neuropathy (ageing of neuropathy target esterase) and the development of pathology is very similar across species, although the doses required and the motor manifestations of the effect are more variable. However, it is difficult to extrapolate from hens, as a result of greater toxicokinetic variability and uncertainty in hens than in conventional laboratory animals such as rats. An example of this is the difference between genetically dissimilar chickens in the doses of OPs needed to cause OPIDP (de Oliveira *et al.*, 2002).

Threshold of Toxicological Concern (TTC)

3.42 The aim of the threshold of toxicological concern (TTC) approach is to allow preliminary risk characterisation of chemicals for which there are few or no toxicity data. The TCC approach might be more useful for contaminants or pollutants than for regulated chemicals, such as pesticides or food additives, as there is less opportunity to obtain toxicological data. The TTC is a conservative, precautionary approach that can be used when experimental data are scarce, which allows risk managers to identify a level of human exposure that is unlikely to cause harm. In general, the extent of toxicity testing considered to be necessary for risk assessment is related to the extent of human exposure, with a full database necessary for a commonly used food additive but less extensive data for a chemical for which there is lower exposure (WHO, 1987). The TTC balances an absence of chemical-specific toxicity data against a level of exposure that is so low that toxicity is unlikely to occur. TTC values are based on analyses of the distribution of toxicological potencies for all compounds with similar chemical structures that have been evaluated for general toxicity and carcinogenicity. By grouping chemicals into broad categories on the basis of a number of structural features, different thresholds can be identified for the different groupings. Exceptions for which a practical TTC cannot be set are dioxin-like compounds, heavy metals (because they were not included in the database used to derive the TTC values) and very potent genotoxins (aflatoxin-like, azoxy- and N-nitroso- compounds) (Kroes *et al.*, 2004). Variability and uncertainty are taken into account since the established risk assessment approaches (linear low-dose extrapolation or the use of uncertainty factors as appropriate) are incorporated into the derivation of the TTC values. Based on structural considerations, a decision tree has been developed which allows safe exposure levels to be established for compounds for which there are few or no data. The TTC approach has had greatest use in the risk assessment of the thousands of flavouring agents that are used at very low levels. The decision tree proposed by Kroes *et al.* (2004) might be useful for potentially genotoxic compounds to which exposure cannot be avoided (e.g. some contaminants) and for which a carcinogenicity study is not available so far. (The decision tree of Kroes *et al.*, 2004, is reproduced at Appendix 6.)

Present regulatory approach to vulnerable subgroups in the population

Embryo, fetus and children

3.43 At present, developmental toxicity studies are carried out for most regulated chemicals. Such studies investigate the potential consequences of exposure of embryos and fetuses *in utero* and a (rodent) multigeneration study evaluates the ability of exposed offspring to develop, mature, mate and suckle their own offspring. A further regulatory development, specifically related to the nervous system, is the developmental neurotoxicity study (Chapter 9), which is now sometimes required by regulatory authorities.

3.44 There is often particular concern over the health risk to infants and children exposed to potentially harmful substances. One of the reasons for this is that a serious adverse effect that would have consequences on the affected person for the rest of their life would affect more years if the exposure occurred during childhood than if it occurred in adult life. In addition to this there is concern that infants and/or children might be more susceptible to some chemicals than adults. A proposal was made in the

Food Quality Protection Act in the United States (USA, 1996), that an additional uncertainty factor should be required for the calculation of health-based guidance values/reference doses of pesticides for infants and children. Whether or not this is required partly revolves around the problem of whether the 10-fold intraspecies default factor is adequate to cover pregnant mothers and their embryo/fetuses, infants and young children. An extra uncertainty factor may not be needed if age-related differences are tested for adequately in the animal species (Renwick *et al.*, 2003). The particular concern about pesticides that led to the USA's Food Quality Protection Act requiring the 10-fold extra uncertainty factor might have been the possibility that effects on the nervous system (and some other organ systems) that in adults might be reversible could, in immature organisms be irreversible (for review see National Research Council, 1993). This would be an example of using a larger uncertainty factor because of the severity of the adverse effect. In this context, it should be noted that many pesticides, especially insecticides, are neurotoxic. However, it has been pointed out that in long-term studies, dietary intake in young animals is typically about twice what it is in adults and therefore an extra two-fold safety factor is already incorporated in the risk assessment in respect of the young (Luijckx *et al.*, 1994). The issue of how to deal with the risk to vulnerable subgroups is covered in more detail in Chapter 4.

Short-comings of present methodology for Risk Assessment

3.45 Variability and some degree of uncertainty cannot be avoided. However, efforts can be made to reduce uncertainty. Toxicological studies in animals are performed under tightly-controlled conditions specified by test guidelines that are designed to minimise some of the physiological variation that may affect the identification and characterisation of a hazard and its dose-response relationship. Age and sex of test and control groups must be matched to minimise such factors interfering with hazard identification.

3.46 In this report recent developments in hazard identification and characterisation are discussed. Present test methods may not be adequate to detect hazards in some areas of potential concern, such as developmental neurotoxicology and endocrine disruption. These areas of toxicology are discussed in Chapters 9 and 10, respectively. Furthermore, there is a need to consider if other concepts and approaches to uncertainty and variability in data used for risk assessment improve the handling and transparency of risk assessment. Such methods include physiologically-based pharmacokinetic (PBPK) modelling, where data are available, the replacement of the NOAEL with the benchmark dose and refinement of uncertainty factors (see Chapter 12), probabilistic approaches and Bayesian methodology. Furthermore, the value of more structured literature reviews and acquisition of data in hazard identification and characterisation need to be considered.

Risk Management

3.47 As has been stated, risk assessment, risk communication and risk management are the three components of risk analysis. A risk assessment that concludes there is a risk to public health at existing or proposed levels of exposure to a substance indicates the need for risk management action. The risk assessor should always try to communicate and explain the variability and uncertainty of a risk assessment to the risk manager. Risk management procedures may include the establishment of maximum use levels in different foods, measures to reduce exposure, setting a maximum residue level/limit (MRL) or refusal to allow a regulated chemical on to the market. Risk management decisions often need to take into account aspects

beyond the risk assessment, such as proportionality of the action and availability of control measures. Risk managers need to consider the possible new risks that might result from the introduction of measures to deal with a source of risk. For example, the adverse effects of chelating agents, that are used to remove radioisotopes from the body, have to be balanced against the risk from the radioisotope remaining in the body. Benefits should be balanced against risks, eg. the nutritional advantage of eating oily fish from omega-3 fatty acids compared with the risk from the presence of dioxins in oily fish (for further details see SACN/COT, 2004). It should be recognised that there might be uncertainty about the benefits as well as about the risks. Risk managers also need to communicate the risk and its management to all stakeholders, including the public.

4 Variability In Hazard Characterisation – General Considerations

Introduction

4.1 The term variability, as used in Chapters 5 and 6, is sometimes called type A uncertainty, aleatory uncertainty, inherent uncertainty or irreducible uncertainty. The term "variability" is preferred because it can be reproducibly quantified and as such is not uncertain. A variable quantity represents true heterogeneity amongst individuals and thus may have a range of different values. This heterogeneity has to be considered in setting to guidance values to ensure that they accommodate the natural divsersity of populations. Examples of this heterogeneity include physiological conditions and states, e.g. pregnancy, the functional maturation of organs, body weight and composition, respiratory rate and food consumption. Thus variability in toxicological responses between species and between individuals arises from inherent biological differences between species and individuals. This variation in the human population cannot be reduced and has to be accommodated in hazard characterisation. Attempts are made to minimise within-species and within-strain variability in toxicological studies on animal models, e.g. by using defined or in-bred strains where available, to improve the ability of the study to detect and characterise effects and to reduce the statistical variance in the dose response. The impact of the within-species variability in a study can be minimised by making the exposed and control groups as similar as possible both in genetic make-up and environmental conditions, eg. by randomising the allocation of animals to different groups and housing conditions during the study. A potential disadvantage of such tight controls of experimental conditions is that this approach reduces the chance of detecting an adverse effect that occurs only in a sub-group of the experimental animals. The use of larger groups of more outbred animals might increase the chance of detecting such groups, but this could not be guaranteed. .

Sources of variability

4.2 The major sources of variability in toxicological assessment relate to the use of animals for hazard identification and characterisation and the variation between humans. These two areas, species differences and human variability are the foci of much of the following discussion. Historically they have been taken into account by the use of uncertainty factors (see Chapter 3). Lack of information about the relevance of study design and animal model as well as high dose extrapolation is a source of uncertainty, rather than variability. Other sources of variability in animal studies relate to factors such as diet and housing, which can be reduced by standardisation. Variability between species and strains is discussed further in paragraph 5.1.

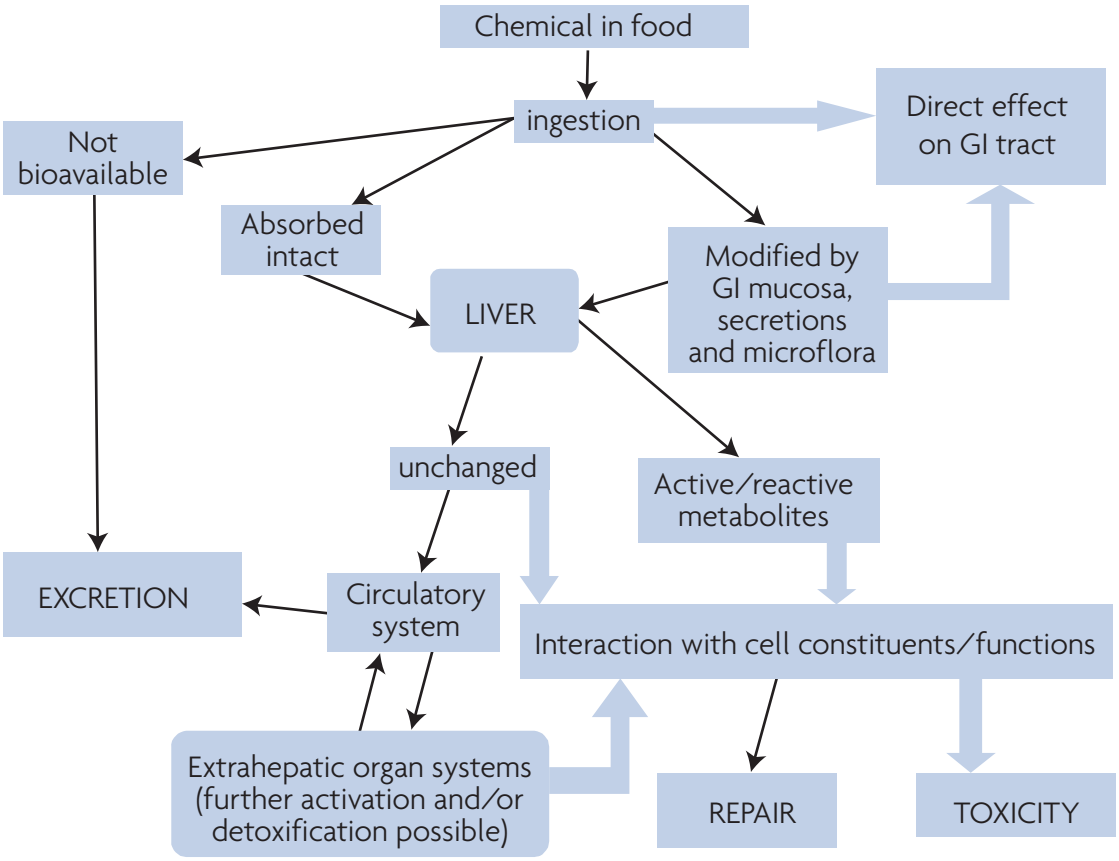
Toxicokinetics

4.3 The sequence between exposure to a chemical and the generation of an adverse effect can be divided into two main aspects: toxicokinetics, the effect of the body on the chemical and delivery of the active form of the chemical to its site of action (see Figure 4.1), and toxicodynamics, the response at the site of action.

4.4 Toxicokinetics, in the context of chemicals in food, is the study of the processes of intestinal uptake and transfer (ie. absorption) of substances by the body, the biotransformation such substances undergo, the distribution of the substances and their metabolites in the tissues, and the elimination of such substances and metabolites from the body (WHO, 1994). Both the amounts and the concentrations of the chemical itself and its metabolite(s) are studied, and the changes in concentration in blood and body tissues after

a dose can provide important information for risk assessment. Toxicokinetic studies are especially helpful for resolving the intricate relationships between the amount given or the intake (the external dose), the amount entering the body (the internal dose, i.e. the body burden or absorbed dose) and the amount at the site of toxicity (the target-organ dose). The term "exposure" is also used, often loosely and with several different meanings such as the total external dose from all sources/routes, for the internal dose to a target tissue. Toxicokinetics provides the link between the external dose of the toxicant, the levels in body tissues over time, and the production of adverse health effects. The possibility of generating relevant kinetic data from studies at non-toxic doses in humans means that issues of variability between species and between individuals can be investigated directly *in vivo* or by the use of *in vitro* data.

Figure 4.1: The toxicokinetics and toxicodynamics of chemicals in food



Note: the broad arrows represent routes leading to toxicity.

Toxicodynamics

4.5 Toxicodynamics is the process of interaction of chemical substances with target sites and the subsequent events leading to adverse effects (WHO, 1994). Each of the stages in toxicokinetics and toxicodynamics is a potential source of variability and uncertainty.

Other sources of variability

4.6 Information on variability in toxicokinetics in humans is mostly available from studies on therapeutic drugs, because controlled human studies are required as part of the approval process. Information on toxicodynamic variability also comes from studies on therapeutic drugs (Evans and McLeod, 2003) and also recreational drugs and, to a lesser extent, occupational exposure to industrial chemicals. This is because these are the situations involving high exposure levels where some individuals may experience adverse effects. Chapters 5 and 6 therefore cite examples of studies on these types of compound, although there may be some uncertainties with respect to the relevance of acute effects to long term, low level exposure to chemicals in food. Data for therapeutic drugs are useful because many of the underlying metabolic and physiological processes are relevant to low molecular weight organic chemicals present in food.

4.7 Sources of variability may be physiological, e.g. age, nutritional status, obesity and exercise, disease related (e.g. diabetes mellitus), or genetic, i.e. species, strain, sex, polymorphisms (genetic variants where the less common allele appears in at least 1% of the population). Characterisation of the human genome and emerging techniques in molecular biology are increasingly enabling identification of genetic variability, and this is a subject of numerous research publications. Chapters 5 and 6 therefore describe such studies with consideration of their relevance to the risk assessment process. Polymorphisms are particularly relevant to interindividual variation in the human population. Clearly, these genetic factors will apply throughout an individual's life, whereas physiological and environmental sources will vary at different times and lifestages.

4.8 Variability may also relate to environmental and social factors – e.g. co-exposures, smoking and alcohol and other recreational drugs.

Vulnerable subgroups

4.9 Certain groups in the population may have the potential to be particularly vulnerable to chemicals in food. One that is of particular concern is that comprising the embryo, fetus and child. Present regulatory responses to these concerns are discussed in Chapter 3.

Embryo, fetus and children

4.10 Particular consideration is often given to possible effects of exposure to the fetus and children, because of the potential long-term impact of adverse effects. There are numerous examples of toxicants that are more toxic to the embryo and fetus than to adult humans. Children are commonly exposed to higher amounts (expressed on a bodyweight basis) of chemicals in food because, adjusted to their bodyweight, their energy and nutritional requirements are greater than those of adults. This difference in intake via food and the potentially greater exposure to chemicals from non-food pathways since young children spend more time on the floor have to be taken into account under the exposure assessment aspects of risk characterisation (and therefore are not considered further in this report).

4.11 Chemicals may act directly on the embryo/fetus or indirectly by producing maternal effects. The former will depend upon the chemical gaining access to the embryo/fetus, and this in turn is largely a function

of (low) molecular weight and (high) lipophilicity, because high molecular weight or ionised compounds will cross the placenta poorly. The nature and prevalence of the effect will depend on the developmental stage concerned (Schardein, 1993). Exposure very early in gestation might kill the embryo. At certain stages of pregnancy, structures such as the nervous system and endocrine system are developing rapidly and exposure may produce irreversible developmental abnormalities (see review by Weiss, 2000).

4.12 In the past, some particular susceptibilities were missed in toxicological assessments (e.g. the human developmental toxicity of methylmercury, thalidomide and valproate). This was because the assessments were based on a restricted testing scheme that did not detect some hazards. Current testing guidelines would have identified these susceptibilities. The testing schemes now used for regulated chemicals take some account of special susceptibilities. However, it is not possible to predict human adverse effects with total accuracy using any test system. For example thalidomide has very limited teratogenic potential in most species used for toxicity testing. In rabbits thalidomide only induces malformations at high exposures that also produce much fetal death, and it only produces phocomelia (limb abnormalities) in primate species, by a mechanism that is still not understood (Kalter, 2004). Hence it is not wise to assert that the best modern testing regimens would not miss such agents nowadays. Nevertheless, safety testing can only use the best test systems currently available. Post-marketing surveillance and pharmacovigilance systems are used in order to detect any adverse effects that were not foreseen by the pre-marketing safety testing of regulated substances. Regulatory authorities respond by taking measures (eg. withdrawal from sale) to limit the amount of harm that might be caused by unexpected adverse effects on the rare occasions that they occur.

4.13 The concerns regarding exposure of children to toxicants are similar to those concerning the embryo and fetus, in that systems (e.g. the nervous system, endocrine system, and immune system) are immature and that adverse effects may disrupt the development and maturation of systems and that effects that in adults might be reversible are not so in children. In the case of breast-fed infants, the blood-milk barrier provides some degree of protection, however lipid soluble compounds such as organochlorine contaminants partition into the fat of breast milk.

4.14 Infants may be sensitive or resistant because of immaturity of enzymes and other processes involved in the elimination of chemicals (toxicokinetics), whilst infants and young children may show increased sensitivity at receptors or other macromolecules where toxins exert their effects (toxicodynamics). Renwick *et al.* (2000) reviewed published toxicity data from studies *in vivo* and concluded that neonatal rodents were not necessarily more sensitive than adults (although they sometimes were) and that although there was the possibility of greater sensitivity in the neonate there was also evidence of more efficient repair and recovery mechanisms. Renwick *et al.* (2000) concluded that the available data did not provide a scientific rationale for an extra uncertainty factor for infants or children based upon any inadequacy of the default inter- and intraspecies uncertainty factor. The uncertainty factors for extrapolating from adult animals to adult humans are also appropriate for extrapolating from young animals to young humans.

4.15 There is concern that exposure to environmental agents in early life might have a role in the pathogenesis, amongst other things, of developmental abnormalities affecting neurodevelopment and behaviour, endocrine and immune function, and carcinogenesis. Whereas it is true that such outcomes are

particularly concerning, this does not necessarily mean that the developing human, both *in utero* and post delivery, and possibly preconception, has an increased susceptibility to environmental hazards. Such concerns do, however, emphasise the need for transparent systematic risk assessments for environmental agents that are sensitive to the vulnerability and uncertainty associated with the developing and maturing organism.

4.16 Growth, changing body composition, and differentiation of tissues and organs in early life, childhood and adolescence may lead to a different susceptibility to environmental agents compared with adults, but although in some instances the consequences of exposure are more severe this does not necessarily imply that the threshold of effects will be lower. Young animals, including humans, may be able to metabolise some agents better than do adults, and have a better ability to recuperate or compensate for adverse events than adults in whom the capacity for architectural and functional regeneration of organs and tissues is reduced. For example, young animals, rodents in particular, are more sensitive than adults to morphine, tetracycline, novobiocin, atropine, organophosphates anticholinesterases, and histamine but less sensitive to codeine, strychnine, isoniazid, methotrexate (Done, 1964). Another example of reduced sensitivity in the young is paracetamol, which is discussed in paragraph 5.14 and 11.7.

4.17 Animal studies may enable assessment of specific aspects of variability and uncertainty in the identification of hazards and of their assessment, but they may not be good models for developmental outcomes, particularly neurodevelopmental outcomes (see Chapter 9).

Influence of Body Composition and Function

4.18 The systemic metabolism and compartmentalisation of agents in the embryo and fetus, and during early life, particularly the first year, differ from the equivalent parameters in adults for a variety of reasons. The size of the lipid-soluble pool is relatively larger in immature animals than in adults, and this has particular implications for neurodevelopmental hazards (Chapter 9). In early life, the potential metabolising systems in the liver, placenta, intestinal mucosa, and kidney may differ from those in later life. This has been variably characterised. There might be substantial metabolism of xenobiotics by the maternal liver, before they reach the placenta or the fetus. In the fetus, however, the role of the liver in xenobiotic metabolism is secondary to that of the placenta. Hepatic metabolism is further limited in the fetus because in the fetal circulation blood returning from the placenta bypasses the portal circulation. This represents the preferential direction, in the fetus, of oxygenated blood to the brain, which is another factor in any possible susceptibility to neurodevelopmental outcomes. Until about six years of life the human brain and its lipid compartment is relatively larger than in adults.

Key issues/Conclusions

4.19 There is a need to understand if a developing human has a distinctive susceptibility to toxicants compared with adults, and whether any difference is reflected adequately by age-related differences in the test animal species. This information would allow assessment of the adequacy of current uncertainty factors as they are applied in risk assessment and setting of exposure limits.

4.20 Ideally, data would be available on the dose-response for all toxicological effects in all age groups. However, it is not always practicable to generate such data and some extrapolation across age groups may

be necessary. An additional x10 factor is probably unnecessary in many cases, such as children, since novel or irreversible toxicity does not necessarily mean that the threshold for effects of agents is lower. It is always better to determine safe exposures directly, rather than by using adult end points and applying a uniform safety factor. The necessity for an additional safety factor should be decided on a case-by-case basis. For instance, in a case in which it is clear that there is a common mechanism (eg. cholinesterase inhibition) for both developmental neurotoxicity and acute neurotoxicity in the adult, there is no need for an additional safety factor unless required for toxicokinetic reasons.

4.21 The elderly may be more susceptible to toxicants by virtue of impaired function of organs such as the kidney. Failing health often means that the elderly are given several different medicines, some of which might interact in complex and unpredictable ways and contribute to the uncertainty about the responses of the elderly to exposures to substances. With an ageing population, the health status of older people can also have major impacts on society and risk managers need consideration of this sub-group in risk assessments. However, the elderly are not a homogeneous group and those most likely to succumb are the frail elderly. There is a need for better characterisation of the uncertainties related to chemical risks to the over sixties.

4.22 The tests that are routinely carried out on regulated chemicals should detect most of the toxicological effects of a chemical, including those that occur late in life. However, it is conceivable that certain types of toxicity might not be readily detected. For instance, a developmental toxicity that was manifested only late in life would not be identified by a multigeneration reproduction study, a developmental toxicity study, or any other of the tests that are routinely performed.

5 Sources of Variability in Toxicokinetics

Species and strain Differences

5.1 In most cases, toxicological data from animal studies are used as the basis for risk assessment. Interspecies differences in toxicokinetics are important when effects in animals are used to establish safe levels of intake in humans. Species differences in toxicokinetics can affect each of the main processes, i.e. absorption, distribution, metabolism and elimination. There can also be marked variations in susceptibility between strains within a species, and an understanding of the mechanisms involved in this can aid extrapolation to man (Pohjanvirta *et al.*, 1999). It has been proposed that replacement of a single strain of outbred rodent with an equivalent number selected from several different inbred strains may provide a more robust model of the genetic heterogeneity present in humans (Festing *et al.*, 2001), although the appropriate uncertainty factors for such a design have yet to be defined. There would also be practical problems in maintaining sufficient stable inbred strains.

Absorption from the gut lumen and metabolism by the gut flora

5.2 The gastrointestinal micro-organisms are collectively metabolically active and those in the human distal bowel have an important role in the degradation of substances that have escaped digestion and absorption in the the upper intestine. Some of these activities are clearly relevant to the metabolism of chemicals in food (Paragraph 5.24) but there is little systematic information on the effect of the intestinal microflora on the fate and systemic uptake of chemicals in foods. Differences in pH and gut microflora within the gastrointestinal tract can result in differences in absorption and metabolism between test species and humans. For example, the gastric pH in rats is about 4-5, compared with about 1-3 in humans, and the higher pH results in a significant bacterial flora in the upper intestine of rats. Lipid soluble compounds that are absorbed in the upper intestine could be subject to bacterial metabolism in rats but not humans. In addition, rats have a large caecum, which is a major site of bacterial metabolism in the lower bowel.

Distribution

5.3 The tissue distribution of chemicals is determined by a large number of factors, including the physicochemical properties of the molecule and the body composition of the exposed organism (e.g. the amount of fat). In some cases the route of exposure may influence distribution. Because the structure and composition of body tissues in other animals and humans are similar, there are not major differences in distribution for many chemicals, once differences in body weight have been allowed for. There is a good correlation between the apparent volume of distribution (a measure of the extent of tissue distribution) and body weight (Boxenbaum, 1984; Bachmann, 1989), and inter-species scaling is carried out assuming that scaling generally is a function of body weight raised to the power of one. However, there are sometimes major interspecies differences in the extent of plasma protein binding and these differences should be taken into account when scaling. In addition, for some chemicals, distribution is influenced by active transport into or out of cells. This is mediated by influx or efflux transporters and can show marked differences between species.

Metabolism and elimination

5.4 The metabolism and elimination of chemicals usually entails two phases. The first is the enzymatic alteration of the chemical and the second phase is the formation of a complex (conjugation) of the chemical or more usually the products of the first phase of its metabolism with an endogenously synthesised compound to form a product that can can excreted by the body. Enzymes involved in these processes are called Phase I and Phase II enzymes. The products of Phase I and Phase II metabolism are polarised water-soluble entities that can be eliminated from the body by the kidneys or via the hepatobiliary tract. Polymorphisms of these enzymes, particularly the Phase I metabolising enzymes, can cause variability in the metabolism and elimination of chemicals.

5.5 The complexity of the overall detoxification and elimination systems partly explains the difficulty in reaching generic conclusions about how these affect risk, particularly in humans. A major and consistent quantitative species difference, which affects the elimination of most chemicals, is the relatively greater cardiac output and perfusion of the liver and kidneys in smaller animals. The higher perfusion rate enhances delivery of the chemical to the organs of elimination, the consequence being a shorter half-life compared with humans. More rapid elimination results in less accumulation during daily intake, and a lower body burden at steady-state for a given daily dose. In addition, the rodent liver has higher enzyme activity for many of the major pathways of foreign compound elimination than does human liver. Inter-species scaling for elimination processes has been proposed using body weight to the power 0.75 (Bonati *et al.*, 1985), and metabolic processes scale to surface area rather than body weight (Calder, 1981; Boxenbaum, 1984; Bachmann, 1989).

5.6 In addition to the quantitative differences outlined above, there may be qualitative differences in the enzymes themselves. Some human cytochrome P450 mono-oxygenase enzymes (CYPs[2]) appear to differ considerably from the corresponding rodent proteins in their specificity for compounds despite their sequence similarity (Chang and Waxman, 1996). However, this is often a consequence of the complexity of the subfamily involved, members of which can display marked species and tissue differences in their expression. An example can be found in enzymes of the CYP2A subfamily. In mouse and human liver, the respective members of the CYP2A sub-family, CYP2A5 and CYP2A6, catalyse the 7-hydroxylation of coumarin. In the rat, CYP2A1 is expressed in liver. This enzyme, though closely related structurally to the CYP2A forms in mouse and human, does not catalyse coumarin 7-hydroxylation but does catalyse the 7α- and 15α-hydroxylation of steroids. In the mouse, a second form of CYP, CYP2A4, catalyses these reactions and is expressed in liver. In the rat, the form of CYP orthologous to mouse CYP2A5 and human CYP2A6 is CYP2A3. This is expressed only in extrahepatic tissues, such as the olfactory mucosa. This enzyme has high coumarin 7-hyroxylase activity. In the mouse, CYP2A4 and CYP2A5 share very high homology (98%), yet differ markedly in their substrate specificity.

2 "CYP" denotes the family of microsomal cytochrome P450 mono-oxygenase enzymes. Sub-families of these enzymes are indicated by "CYP" followed by a number and a letter (eg. CYP2A). Individual enzymes are indicated by "CYP" followed by a number, a letter and a number (eg. CYP2A5).

5.7 Phase II conjugation reactions also differ between species: for example. Steensma *et al.*, (1994) demonstrated that 7-ethoxycoumarin was converted into equal amounts of glucuronide and sulphate conjugates in cynomolgus monkeys, guinea pigs and humans, whereas in the rat it was predominantly sulphated.

5.8 The actions of known inhibitors and inducers of foreign compound metabolism can vary between species. The distribution of xenobiotic metabolising enzymes (XMEs) can differ between test species and humans, as indicated above by the example of the CYP2A subfamily. CYP1A1 is not normally expressed, or is present at only very low levels, in the tissues of experimental species and humans. However, following exposure to an inducing agent, such as a polycyclic aromatic hydrocarbon, CYP1A1 levels are increased in liver and extrahepatic tissues such as the lung of rodents and cynomolgus monkeys but primarily in extrahepatic tissues of humans and marmosets with little or no induction in liver. Studies of lung tissue from smokers and non-smokers have confirmed that smoking increases the activity of CYP1A1-related activities in this tissue (Doehmer *et al.*, 1993). In most species, including human, CYP1A2 is constitutively expressed, almost exclusively in the liver. However, in the cynomolgus monkey, there is no constitutive expression of CYP1A2 and, unlike in rodents, marmosets and humans, it is only very weakly inducible by agents such as polycyclic aromatic hydrocarbons and dioxins (Edwards *et al.*, 1994).

5.9 There are well-established species differences in the molecular weight thresholds that determine whether xenobiotic excretion is renal or hepatobiliary. This is due to differences in the expression and properties of the transporters located in the canalicular membrane, which determine the composition of bile (Ishizuka *et al.*, 1999). These transporters include P-glycoprotein (ABCB1), multidrug resistance protein 2 [MRP2 (ABCC2)], the bile salt export pump [BSEP (ABCB11)], and the breast cancer resistance protein [BCRP (ABCG2)] (see Chandra and Brouwer, 2004). The bile is the main route of excretion for large molecules; for anionic compounds the molecular weight threshold for significant biliary excretion is 325 in rats but is 500 in humans (Hirom *et al.*, 1976). A consequence of this is that chemicals and their conjugates with a molecular weight between 325 and 500 may undergo significant enterohepatic circulation in the rat, but not in humans. There are species differences in the clearance of chemicals eliminated in urine, but these are often due to differences in glomerular filtration rate and renal blood flow (Walton *et al.*,2004).

5.10 Species differences must be taken into consideration when human risk assessment is based on toxicological data obtained from studies carried out in laboratory species such as rodents. Ideally toxicity studies would be conducted in the animal species exhibiting toxicokinetics for the substance of interest that most closely resemble those in humans (especially similarity in the metabolites formed); however, in practice this is not always feasible, and rodents and a restricted range of other species are commonly used. This is based on availability and practicability and the existence of an extensive historical database, particularly with respect to histopathology. The final choice of species will nevertheless be guided by similarity in toxicokinetics of the test species to humans, to the extent possible.

5.11 When substances are given to food producing animals (eg. veterinary drugs, feed materials) human consumers might be exposed to both the substance administered and its metabolites when eating foods derived from the treated animals (eg. meat, milk, eggs). In such cases, the role of metabolism by the food-producing animal and its gut microflora needs to be taken into consideration when evaluating consumer safety.

Physiological Factors Affecting Human Variability

5.12 There are many sources of variability in the toxicokinetics of compounds in different human individuals that need to be taken into consideration when describing uncertainty in the risk assessment. Outlined below are the major sources of variability in kinetics. Many of the examples are drawn from the pharmaceutical literature, because that is where there are the greatest number of data, however such examples are relevant to many other xenobiotics.

Age

5.13 Age has an important influence on hepatic metabolism and renal excretion, both of which are major pathways of foreign compound clearance. Age-related changes in kinetics occur both in laboratory animal species and in humans. The kinetics of water soluble agents and metabolites might be limited by the slower renal elimination of metabolites and toxicants of the maturing kidneys during the first two to four months of life, and by different binding characteristics of protein binding in the circulation in the same period. Toxicity studies in animals are designed to cover the extremes of age by means of developmental studies and chronic toxicity studies. The following text relates to the influence of age in humans, since this is a major source of human variability in metabolism. Brent (2004) pointed out that although there was a vast number of animal toxicology studies carried out on pregnant animals and adult animals, there were few animal studies utilising newborn, infant, and juvenile animals. Reviewing relevant data, Brent (2004) concluded that although many studies showed that infants and developing animals might have difficulty in metabolising drugs and were more vulnerable to the toxic effects of some chemicals, there were exceptions that indicated that infants and developing animals might indeed be less vulnerable than adults to some drugs and chemicals. Thus the generalisation that developing animals are always more sensitive to environmental toxicants is not valid. None-the-less, increasing understanding of metabolic programming, epigenetic influences and other influences of early life events generate interest in the possibility that problems seen in adults affecting growth, endocrine function, neurobehavioural endpoints, and oncogenesis might be related to exposures to chemicals including drugs during development.

5.14 There is a low elimination capacity in human neonates compared with adults, primarily due to immaturity of metabolising enzymes, especially some of the CYP enzymes and glucuronyl transferase and also of renal function. Adult levels are reached by 6-12 months of age (Dorne and Renwick, 2004). Immaturity in metabolic pathways in neonates is commonly considered to result in increased susceptibility to toxicants, although a relative functional deficiency of some metabolising systems in the liver may be at least partially offset by the relatively larger size of the organ in early life. However, under some circumstances, the immaturity of certain metabolic pathways in neonates may actually result in a lower susceptibility to certain toxicants. For example, high levels of paracetamol can cause fatal hepatotoxicity in adults, but babies born to mothers with high levels of paracetamol, which will also have elevated paracetamol levels in their blood, do not sustain liver damage, probably because of low hepatic levels of CYP1A2 and CYP2E1, the enzymes that metabolise paracetamol to the active hepatotoxin N-acetyl-p-benzo-quinoneimine (Rumack, 1984: Lesco and Mitchell, 1999). It has been observed that the half-life of paracetamol in blood of the offspring of poisoned mothers is longer than in the mother (Roberts *et al.*, 1984), presumably because of lower conjugation capacity.

5.15 Not all enzymatic pathways are immature at birth and the isoenzyme pattern may change rapidly post-partum. For example, CYP3A7 is the major form of CYP3A in human fetal liver whereas CYP3A4 is the main form present in adult liver (Pelkonen *et al.*, 1998; Wrighton *et al.*, 1996).

5.16 In the case of the synthetic pyrethroid, deltamethrin, toxicokinetics vary markedly with age. Thus, although lethality in rats was found to occur at closely similar brain concentrations of the insecticide in both 72 day old adults and 11 day old neonates, these concentrations were achieved at 16-fold lower oral doses in the neonates (Sheets *et al.*, 1994). At the intermediate age of 21 days, pups were 7 times more sensitive to the lethality of deltamethrin than adults. In contrast to this, at sub-lethal doses that did not saturate metabolic capacity there were no differences between neonates and adults. Hence, there was no need to propose any inherently greater susceptibility of neonates to the acute toxicity of deltamethrin, but the capacity of young rats for metabolic detoxification of large quantities of deltamethrin was clearly markedly less than that of adults. Unfortunately, such informative toxicokinetic data in neonates is rarely available.

5.17 Elimination by children often exceeds that by adults when expressed on a body weight basis, largely because of the proportionally higher hepatic and renal blood flows.

5.18 Renal and some hepatic functions tend to decline in humans with age beyond about 20 years by approximately 1% per year, resulting in slower elimination of foreign compounds by the elderly. Slower elimination of medicines has also been reported between young adults and elderly individuals. For example, the elderly show impaired elimination of the anti-arthritis drug benoxaprofen due to a decline in renal function with age and an increased risk of adverse effects (Koch, 1982). Chronic animal studies include aged animals, and therefore risk assessments based on such data will have taken into account the influence of ageing processes.

Other physiological sources of variability

Body weight and weight change

5.19 Large weight differences can contribute to variability in toxicokinetics, particularly in the pattern and extent of distribution immediately after ingestion of a chemical. For example, in rats, after administration of a single dose of TCDD, it takes about 3 days for blood and adipose tissue to reach equilibrium, and even longer would be required for humans. The extent of distribution influences the rate of elimination, elimination of chemicals that accumulate in adipose tissue tending to be slower than that of those that do not. Distribution is of less importance during repeated intake, because the steady-state concentrations in blood and tissues depend on the rates of absorption and of clearance from the blood, which is determined by processes such as metabolism and excretion, and not tissue distribution. The tissue distribution, which can be influenced by body composition, determines the relationship between the concentrations in blood and tissues and the total body burden. The steady-state body burden, which has been used in the risk assessment of TCDD, is determined by the steady-state concentration in the blood/plasma and the extent of tissue distribution, as indicated by the apparent volume of distribution, which can vary between species and between different individuals.

5.20 Changes in weight, for example during rapid weight loss, result in lipid mobilisation, with the release into the blood of chemicals that have accumulated in adipose tissue during chronic intake. This redistribution of chemicals stored in adipose tissue can result in a small increase in the concentrations present in non-adipose tissues and an increase in risk of acute effects. This may be of greatest importance for chemicals with very long half-lives due to a large apparent volume of distribution (Vd), such as dioxins, where loss of weight in the months preceding or during pregnancy could produce a sustained increase in the circulating concentrations to which the developing fetus is exposed, thereby increasing risk.

5.21 During lactation, highly lipid soluble chemicals may be mobilised from lipid stores in the body and accumulate in breast milk, from where they can be transferred to suckling infants by lactational transfer. An example of this is the finding of persistent environmental contaminants such as organochlorine compounds in human breast milk. Also, lead might be redistributed from bone to milk if calcium is redistributed during lactation. Such redistribution of chemicals will result in high and variable exposure of both the neonate and the target tissues of the mother during lactation.

5.22 Morbid obesity has been reported to result in the up-regulation of hepatic CYP2E1, which may be associated with liver disease (Emery *et al.*, 2003). By contrast the activity of CYP3A4 decreases in obesity, while the effect on other forms is inconclusive (Kotlyar and Carson, 1999).

Exercise

5.23 Exercise is another physiological factor that can affect toxicokinetics, although the results are minor and inconsistent (although exercise can greatly increase inhalational exposure). Apart from a change in lean body mass, which could influence distribution, there are no clear trends to indicate that the elimination of chemicals is either increased or decreased in those who undertake regular exercise (Persky *et al.*, 2003).

Changes in gut flora

5.24 The gut microflora can cause reductive and hydrolytic reactions, including certain reactions (eg. nitroreduction) that are not readily performed by systemic or gut mucosal enzymes. Other reactions can also be catalysed, including oxidation and ring scission. Certain species of gut bacteria, such as *Escherichia coli*, produce ß-glucuronidase, which readily hydrolyses glucuronide conjugates of xenobiotics excreted via the bile into the intestinal tract. Depending on its physicochemical properties (pKa and lipid solubility), the liberated aglycone may then be reabsorbed into the systemic circulation. Reductive reactions catalysed by the gut microflora include azo- and nitro-reduction. Enzymes produced by gut microflora can degrade naturally occurring xenobiotics, such as flavonoids. Changes in gut flora will alter these processes and contribute to variability. There are species differences in the composition of the intestinal microflora and in their distribution along the gut lumen. The upper gastrointestinal tracts of humans, dogs and rabbits are almost sterile, while significant numbers of largely aerobic bacteria are present in the rodent stomach and small intestine. The gut microflora has a greater role in digestion and metabolism in animals with a rumen (eg. cattle, sheep, goats) or caecum (eg. horses, rabbits, rodents) than in other animals (eg. humans, dogs, cats, pigs). The site and composition of the microflora can influence the toxicokinetics and toxicodynamics of substances in the diet, and the composition of the gut flora can in turn be affected by diet and disease. This can add to the uncertainty in extrapolating results from one species to another.

Disease processes

5.25 The main diseases that influence toxicokinetics are those involved with the basic processes of absorption and elimination, i.e. diseases of the gastrointestinal tract, liver and kidneys. The tissue distribution of chemicals can be affected by diseases that alter the concentrations of plasma proteins, such as albumin, which is decreased in liver disease and renal failure, and α1-acid glycoprotein, which is increased in inflammation. Infections and inflammatory stimuli also cause changes in the toxicokinetics of compounds by affecting the activities and expression of various CYPs in the liver as well as in other tissues such as kidney and brain (Morgan, 1997). In most cases, CYPs and their activities are suppressed, but some are unaffected or induced under these conditions. CYP suppression can increase circulating levels of the substrate and result in increased clinical toxicity of drugs with a low therapeutic index.

Environmental, Social and Dietary Factors Affecting Human Variability

5.26 Environmental factors such as co-exposure to drugs or other chemicals, the composition of the diet and lifestyle choices can all influence kinetics. Exposure to chemicals other than the one of interest can modify the expression or activity of enzymes of toxicant metabolism. Where compounds are high affinity ligands for an enzyme they may act as competitive inhibitors of that enzyme, for example some flavones inhibit CYP1A2. However, other compounds can inhibit the enzymes non-competitively, in a reversible or irreversible manner. This may be due to tight binding of the compound to the enzyme, for example a number of amino and dioxo compounds are inhibitors of certain forms of CYP by forming tightly bound complexes with them. Other compounds act as mechanism, or suicide, inhibitors of the enzyme. Such compounds serve as substrates and the product is chemically reactive such that it binds to and inactivates the enzyme, often with subsequent degradation of the protein. In the case of CYP, binding may be to the haem moiety or to the apoprotein. For example norethisterone binds to and inactivates CYP haem whilst carbon tetrachloride binds to and inactivates the apoprotein. Such inhibition requires *de novo* synthesis of enzyme before activity is restored.

5.27 Many drugs can inhibit enzymes of drug metabolism, for example ketoconazole and CYP3A4, paroxetine and CYP2D6, disulfiram and CYP2E1, sulfinpyrazone and CYP2C9, omeprazole and CYP2C19. Dietary constituents that can inhibit enzymes of drug metabolism include bergottamin derivatives in grapefruit juice, quercetin (found in apples, onions and tea), certain other flavanoids, 3,3-diindolylmethane (found in cruciferous vegetables) and 8-methoxypsoralen (found in many plant species such as celery, figs, parsnips and limes). In a study using twenty-four healthy adult volunteers the effect of grapefruit juice consumption on the pharmacokinetics of the antihistamine drugs fexofenadine and desloratadine were compared. Treatment with 240 ml of double-strength juice three times daily reduced the rate and extent of absorption of fexofenadine by 30%. In contrast, the bioavailability of desloratadine was unaffected by grapefruit juice (Banfield *et al.*, 2002). This effect was largely due to inhibition of intestinal mucosal CYP3A4.

5.28 Many of the enzymes of drug metabolism are inducible by xenobiotic compounds. This usually involves activation of transcription factors, up-regulation of mRNA and increased enyme expression. However, other mechanisms can be involved, including mRNA and protein stabilisation. For example induction of CYP2E1 often involves post-transcriptional effects. There has been much progress recently in identifying transcription factors involved in the induction of enzymes of drug metabolism. These include several

nuclear receptors, such as pregnane X receptor (PXR), constitutive androstane receptor (CAR), peroxisomal proliferator activated receptor alpha (PPARα), the aryl hydrocarbon receptor (AhR) and nuclear receptor factor 2 (NRF2). Induction involves activation of the receptor, either by direct binding (in the case of PXR, PPARα and AhR) or indirectly (in the case of CAR and NRF2). The receptor then forms a heterodimer with a second transcription factor (retinoic acid X receptor (RXR), in the case of CAR, PXR and PPARα; aryl hydrocarbon receptor nuclear translocator (ARNT) in the case of AhR). The resulting dimer binds to a specific enhancer or response element upstream of the transcription start site and increases transcription leading eventually to more active enzyme. Examples of inducers include terpenoids and dioxins (such as TCDD) which are potent inducers of members of the CYP2 family (CYP2B and CYP2C) and CYP1 family (CYP1A and CYP1B), respectively. Compounds present in certain dietary components can induce enzymes of drug metabolism. These include flavanoids in cruciferous vegetables and polycyclic aromatic hydrocarbons in barbecued food. A number of drugs are enzyme inducers, for example phenobarbitone, carbamazepine, rifampicin and isoniazid. Inducers can also be found in environmental pollutants (e.g. polycyclic aromatic hydrocarbons), occupational exposures (e.g. organic solvents such as acetone) and herbal remedies (e.g. St John's Wort).

5.29 As the above examples demonstrate, both inhibition and induction are selective, restricted to only certain members of a family of xenobiotic metabolising enzymes. However, particularly in the case of induction, the enzymes affected by a single agent may come from different families, e.g. CYP and GST. Hence, the net effect will depend on the relative efficacy of induction of the respective enzymes together with the contribution of the induced enzymes to the overall fate of the active species (i.e. between activation and detoxication).

5.30 Both macronutrients and micronutrients in the diet can affect the activities of xenobiotic metabolising enzymes, although the effects of deficiences or excess are relatively minor except in cases of severe deprivation. Neither vitamin C deficiency nor supplementation in ascorbate non-deficient subjects has any appreciable effect on CYP-dependent activity. In ascorbate-deficient subjects, vitamin C supplementation causes a modest increase in CYP-dependent activity. High doses of vitamin C (>1 g/day) can cause sufficient depletion of intestinal epithelial sulphate levels to increase the bioavailability of compounds normally subject to pre-systemic metabolism by this route, e.g. tyramine. Pyridoxine is a precursor in the synthesis of pyridoxal 5'-phosphate, which is an important co-factor in the enzyme dopa decarboxylase and dietary supplementation with pyridoxine appears to increase this activity (see Wilkinson, 1997). Dietary selenium can increase the activity of some primarily phase II enzymes, including glutathione peroxidase and glutathione S-transferases. In contrast, dietary selenium reduces the activities of some forms of CYP in the rat. The extent to which selenium affects drug metabolising enzymes in humans is not known.

5.31 While the liver is often the main organ affected, there can be important changes in the enzymes of the epithelial cells of the intestinal tract (especially of the small intestine). In rodents, there is good evidence that dietary micronutrients can markedly affect small intestinal CYP levels: iron restriction results in a rapid decline in intestinal enterocyte CYP content to levels that are undetectable and selenium deficiency has similar though less marked effects. Reduction of vitamin A content of the diet increases intestinal CYP-dependent activities. Conversely, dietary supplementation with vitamin A results in a decrease in intestinal CYP-dependent activities (see Kaminsky, 1991). However, the extent to which these changes

occur in humans is not known. There can also be changes in the composition or the gut microflora. Such effects can, in turn, alter the presystemic metabolism of compounds, affecting their bioavailability or the hydrolysis of conjugates excreted via the bile and hence enterohepatic recycling, leading to changes in the toxicokinetic profile.

5.32 Those on unusual or restricted diets, which may include vegetarians or those on low protein diets, also exhibit variability in the metabolism and/or clearance of drugs. For example Caucasians who eat meat regularly have a significantly greater clearance and shorter plasma half-life of some compounds, e.g. the analgesic drug antipyrine, than those who eat meat less frequently such as Asiatic vegetarians. It should however be remembered that ethnic differences may complicate this difference and the magnitude of such effects is often quite small. Nutritional deficiencies, such as occur with pan-malnutrition, can increase the hepatotoxicity of paracetamol due to altered metabolism. Dietary deficiency of calcium can lead to increased absorption of divalent metals such as lead (see review by Ballew and Bowman, 2001).

5.33 Individuals' chosen lifestyles can also influence the toxicokinetics of compounds; polycyclic aromatic hydrocarbons in cigarette smoke induce CYP1A2 in liver and CYP1A1 and CYP1B1 in extrahepatic tissues. Consumption of alcohol (ethanol) over a period of time enhances the expression of CYP2E1 (Lewis, 1997) whereas acute ingestion results in inhibition of the enzyme. Certain recreational drugs can also affect enzymes of drug metabolism. For example, methylenedioxymethamphetamine (MDMA, "ecstasy") is a potent suicide inhibitor of CYP2D6. Tetrahydrocannabinol (THC) present in cannabis can also reduce enzyme activity. Various drugs, such as cimetidine, which is commonly used for treating gastric and duodenal ulcers, inhibit the activity of a number of forms of CYP.

Genetic Factors Affecting Human Variability

Polymorphisms

5.34 A polymorphism is a genetic variant in the DNA sequence where the less common allele appears in at least 1% of a population (Miller *et al.*, 2001). This usually gives rise to a variant of the corresponding protein. Proteins such as enzymes, receptors and transport proteins may all exhibit polymorphism and enzymic and transporter polymorphisms may be sources of toxicokinetic variability (receptor polymorphisms are potential sources of toxicodynamic variability and are dealt with in paragraphs 5.58 to 5.61). Polymorphisms that alter the amino acid sequence of the protein product of a gene and hence the action of the protein are readily understood. In other cases polymorphisms may be found in the non-coding regions of genes, affecting mRNA splicing or the control of transcription. In some cases polymorphisms have been identified which do not directly affect either the primary sequence of a protein or the control of gene expression, yet appear to be related to altered susceptibility to diseases or toxic effects. In these cases it is necessary to consider the possibility of genetic linkage to functional polymorphisms, whereby the non-functional marker polymorphism is inherited in tandem with an as yet unidentified functional polymorphism. It should also be noted that the complete absence of a gene product (called a null genotype) may also be classified as a polymorphism. As genetic polymorphisms can affect either the toxicokinetics or toxicodynamics of xenobiotics, an understanding of genetic polymorphisms is important because it facilitates the process of understanding variability in response within the population. It may, in some cases, be possible to identify and protect a particularly susceptible

subpopulation. It was with this in mind that the Environmental Genome Project (based at the National Institute of Environmental Health, NC, USA) was initiated (Olden and Guthrie 2001; Olden *et al.*, 2001).

Genotyping and phenotyping

5.35 The genotype of an individual refers to his/her genetic characteristics i.e. which alleles are carried. Phenotype refers to the biological expression of the genotype, e.g. the activity of a particular enzyme, level of a receptor or expression of a particular physical characteristic. The methodology used to determine genotypes and phenotypes in a pharmacogenetic setting has recently been reviewed by Daly (2004); key points regarding methodologies are summarised below.

5.36 Many polymorphisms affect the amino acid sequence of a protein, although some affect the non-coding regulatory regions of genes (see paragraph 5.34). Thus, polymorphisms which affect amino acid sequence may be detected either at the level of nucleotide sequence or amino acid sequence. Polymorphisms that affect non-coding regions may be detected at the nucleotide sequence level or by looking at mRNA expression or protein expression. Polymorphisms that affect function may, of course, be detected by measuring the function (e.g. enzyme activity). Techniques that investigate the nucleotide sequence directly are called genotyping methods whereas those that address expression and function are known as phenotyping approaches.

5.37 Traditional methods for examining polymorphisms in toxicokinetics, particularly those in xenobiotic metabolising enzymes, involve measuring enzymatic activity using diagnostic substrates. The advantage of this approach is that it addresses the actual function of the enzyme, but it has become less popular in recent years because it is more labour intensive than genotyping (see below at paragraph 5.38). In addition, phenotyping requires the use of relevant tissue samples (where possible, samples from the target tissue); this has implications in terms of access to material for analysis and the willingness of volunteers to participate in epidemiological studies.

5.38 Genotyping involves direct examination of the altered nucleotide sequences of polymorphic variants. This approach traditionally involved cloning and sequencing the gene of interest from different individuals, but recent developments in technology mean that genotyping is now usually undertaken using polymerase chain reaction (PCR) based methods. These include allele specific PCR and PCR followed by restriction digest to reveal restriction fragment length polymorphisms. A powerful new approach for the identification of novel polymorphic variants involves the identification of single nucleotide polymorphisms (SNPs). Nonsynonymous SNPs result in amino acid changes whereas synonymous SNPs do not lead to alterations in the amino acid sequence of the protein produced. Taking both the synonymous and non-synonymous classes into account, SNPs are found in the human genome at a frequency of approximately 1/1000 base pairs.

5.39 All of an individual's cells (except for red blood cells because they have no DNA) contain the same genomic DNA sequence, so the genotype can be determined by looking at any cell type. White blood cells are usually used for this purpose since these may be obtained by relatively non-invasive methods. However, in order to look at levels of expression and function, it is important to identify and access the tissue of interest. In the case of polymorphisms which affect toxicokinetics, many of which affect hepatic

enzymes, this would entail obtaining liver tissue (i.e. by biopsy), and this is clearly impractical when looking at healthy populations. Attempts have been made to use surrogate tissues such as isolated white blood cells to examine phenotypic variation in the expression of xenobiotic metabolising enzymes. Variable CYP expression has, for example, been detected in leukopheresed white blood cells (Bernauer *et al.*, 2003), but it must be emphasised (and was acknowledged by the authors of this study) that this might not reflect the situation *in vivo*. Indeed, there is evidence to the contrary, ie. that expression in white blood cells does not reflect that in the liver.

5.40 Another issue to be considered in examining phenotype is that the expression of a particular mRNA may not directly reflect the expression of the cognate protein. Many studies now use mRNA-based techniques such as TaqMan "real time" PCR, which is a simple and rapid method for quantifying the expression of a particular mRNA. However, the results of these studies must be examined critically because of the potential that differences in mRNA levels may not be reflected at the protein (and hence functional) level. For example, one study on microsomal epoxide hydrolase looked at 40 human livers (16 female, 24 male), examining variation in enzymatic activity, mRNA expression and protein expression (Hassett *et al.*, 1997). The results revealed an 8.4-fold variation in expression at the protein level; as expected, this correlated with the measured enzyme activity. However, there was 49-fold variability in the expression of the corresponding mRNA and poor correlation between mRNA and protein expression. The lack of correlation was suggested to be due to post-transcriptional mechanisms of regulation such as control at the level of translation.

Correlating genotype and phenotype

5.41 The use of genotyping methods to examine polymorphisms within the population is based upon the assumption that an individual's phenotype is a direct function of the genotype. It is usually the change in function of a gene product that alters risk and this is clearly an important consideration in risk assessment. Thus, differences in genotype without alterations in phenotype usually have no impact on risk, whilst changes in phenotype without genotypic basis can impact on vulnerability. In order to apply the assumption that phenotype is a direct function of the genotype it is necessary to demonstrate a clear correlation between genotype and phenotype. In fact, the relationship between genotype and phenotype is complex and sometimes unclear. This is exemplified by paraoxonase (PON1), an enzyme that hydrolyses OP insecticides and nerve agents (Costa *et al.*, 2002: Mackness *et al.*, 2002). PON1 polymorphisms affect the rate of hydrolysis of some OPs, but currently phenotype is more predictive than genotype of functional capacity to hydrolyse oxons (Costa *et al.*, 2002: Mackness *et al.*, 2002) (see reviews by Costa *et al.*, 1999; Costa *et al.*, 2003). With this enzyme, there are two isoforms of PON1 in humans at the relevant locus: the Arg^{192} (R) and the Gln^{192} isoform (Q). The presence of two alleles within the population means that there are three possible genotypes: RR, QR, QQ. Within each genotype group, however, there is a 15 fold variability in expression, with considerable overlap between different genotypes. Unfortunately PON1 activity is very dependent on reaction conditions in the enzyme assay (O'Leary *et al.*, 2005). These factors mean that the identification of a susceptible population cannot be achieved by looking at the genotype in isolation. PON1 has roles in metabolism independent of OPs. PON1 polymorphisms are associated with differences in the prevalence of adverse outcomes independent of the OP exposure, including coronary heart disease (Shih *et al.*, 2002). This might confound some of the studies in this area.

Polymorphic xenobiotic metabolising enzymes

5.42 Polymorphisms exist among both Phase I and Phase II xenobiotic metabolising enzymes (XMEs). When predicting the consequences of variation in xenobiotic metabolism for susceptibility to toxicity, it is important to understand the metabolic pathways involved and to identify the actual toxic agent. If the parent compound is the toxic agent, then the lower end of the activity range represents a risk factor since the population below the median is at greater risk, whereas if the metabolite is the toxic agent, the top end of the variability range may be at greater risk. The adverse impact of polymorphisms of XMEs that affect metabolism will also depend on how critical the enzyme is in either the bioactivation or in the overall elimination of the compound, and also whether alternative pathways exist.

5.43 Amongst the earliest known CYP polymorphisms is that associated with debrisoquine, an agent used in the treatment of hypertension. Most humans are able to hydroxylate this drug, but about 5-10% of the Caucasian population excrete the drug virtually unchanged and are termed "poor metabolisers" (Gonzalez and Nebert, 1990). These individuals will have higher circulating levels of the active drug when prescribed a clinical dose of debrisoquine and will be more prone to adverse reactions from this or other drugs catalysed by CYP2D6. XME polymorphisms may affect a number of gene products, including several CYP enzymes, xanthine oxidase, alcohol dehydrogenase, aldehyde dehydrogenase, glutathione S-transferases (GSTs), N-acetyltransferases (NATs), UDP-glucuronyl transferases (UDPGTs), choline esterase, phenylacetate esterase, methyltransferase and paraoxonase (reviewed in Daly, 2003) and summarised in Appendix 2.

5.44 The majority of studies on polymorphic variation and risk of toxicity or disease have, for scientific and resource reasons, concentrated on one or two key genes. However, in practice, susceptibility to toxic agents almost always involves many factors. Redundancy within xenobiotic metabolising pathways means that, if the activity of one enzyme is reduced, potential toxic agents may still be eliminated by other pathways. For example, if the polymorphic pathway contributes 50% to overall elimination, even complete deletion of this pathway would result in only a two-fold decrease in clearance. These other pathways may compensate for the loss of activity, or may lead to the generation of a reactive intermediate with enhanced toxic effects. For example, in smokers, various CYPs, GSTM, GSTT, GSTP, NAT1 and NAT2 may exert combined effects on DNA adduct formation and cancer susceptibility. The epidemiological studies required in order to understand the roles of all the possible polymorphic variants of these enzymes would need to be enormous. A number of these polymorphisms appear to have some impact on cancer susceptibility (see review by Thier *et al.*, 2003), although their impact on susceptibility to acute toxic effects is often less well understood.

Cancer susceptibility

5.45 An example that illustrates the role of polymorphic XME in cancer susceptibility is bladder cancer induction by exposure to aromatic amines. Carcinogenic aromatic amines are subject to acetylation by N-acetyltransferases NAT1 and NAT2. A number of methods are available for the determination of NAT2 genotype by PCR, and this has facilitated epidemiological analysis of the NAT2 polymorphism in bladder cancer. Individuals may be classified as either fast or slow acetylators depending on which alleles of NAT2 they carry, and epidemiological analysis has suggested that slow acetylators have an increased risk of bladder cancer. It is believed that the increased susceptibility of these individuals is due to decreased

detoxification of aromatic amines by N-acetylation. A recent meta-analysis considering 2000 cases and 3000 controls indicated that the slow acetylator phenotype causes an increase in risk of ~40% with exposure to aromatic amines (reviewed in Brennan, 2002). This increased risk in slow acetylators of less than 2-fold is adequately covered by the 10-fold uncertainty factor that is commonly used to allow for variability in the human population (assuming that a 2-fold difference in response to the same level of exposure indicates a 2-fold difference in sensitivity).

5.46 The role of NAT2 should not be considered in isolation, since susceptibility to bladder cancer is also modulated by interaction with other xenobiotic metabolising enzymes. The role of the other NAT isozyme (NAT1) is poorly understood: this isozyme, which is also polymorphic, is expressed in the urothelium and has the capacity to metabolise aromatic amines, but it is as yet unclear what the consequences of this polymorphism are for bladder cancer susceptibility. Part of the problem in terms of understanding NAT1 and its effects on susceptibility is that this gene is both genetically polymorphic (having at least 26 allelic variants) and subject to regulation by post-translational mechanisms, with the result that the genotype/phenotype correlation for this gene is difficult to classify (Rodrigues-Lima and Dupret 2004). Furthermore, individuals who have high CYP1A2 activity in combination with the slow acetylator phenotype are thought to have elevated risk of bladder cancer due to rapid activation and slow detoxification of aromatic amines, and the *GST*M1*0 null genotype further increases bladder cancer risk in slow acetylators.

5.47 Occupational exposure to aromatic amines, while being one of the better understood examples of cancer risk factors modulated by polymorphisms in XME, is by no means the only example. Other carcinogens to which workers are occupationally exposed, such as styrene, also undergo polymorphic metabolic activation, the rate of which is modulated by the genotype of the individual, to form genotoxic metabolites (Teixeira *et al.*, 2004). Nevertheless, the extent to which this influences risk has yet to be determined (there is no good evidence that styrene is a human carcinogen). It has been suggested that genotyping should be used to evaluate the individual genetic susceptibility of exposed populations; however, given that potential susceptibility is often mediated by a number of polymorphic genes (in the case of styrene, GSTP1, GSTM1 and CYP2E1) it will prove difficult, if not impossible, to predict susceptibility on an individual basis. Furthermore, the role of the various implicated enzymes must be clarified. One recent study states that the incidence of chromosomal aberrations in workers exposed to benzene is influenced by a subset of XME genotypes including GSTM1 null, GSTT1 null, slow NAT2, variant NADH-ubiquinone oxidase (NQO1), and CYP2E1 Dra1/Rsa1 (Kim *et al.*, 2004); however, it is difficult to understand, for example, how NAT2 genotype can affect susceptibility to benzene given that benzene lacks an amine moiety, unless there is some as yet unidentified endogenous substrate for NAT2 that represents a risk factor!

Susceptibility to the toxicity of industrial chemicals

5.48 Relatively few well-characterised examples of acute toxicity affected by polymorphic XMEs exist in the literature. One of the best to date is that of acrylonitrile, an industrial solvent which, in addition to being carcinogenic, is acutely toxic. There is significant inter- and intraspecies variability in response to the acute toxicity of acrylonitrile (Thier *et al.*, 2001). Acrylonitrile is known to be metabolised via oxidative and reductive routes, mediated by CYP2E1 and glutathione-s-transferases (GSTs) respectively. The reductive route, which leads to the excretion of mercapturic acid in the urine, is the primary detoxification pathway

whereas the oxidative route, generating glycidonitrile and cyanide, is thought to mediate the carcinogenic and acute toxic effects of acrylonitrile. The role of polymorphisms in susceptibility to acrylonitrile poisoning is incompletely understood, but studies on individual cases of acrylonitrile intoxication have provided some clues. An anecdotal report of the effects of acute exposure to high levels of acrylonitrile in two individuals, one of whom was classified as a "non-conjugator" suggested that levels of acrylonitrile in blood were higher in the non-conjugator as was the level hydrocyanic acid (Leng and Lewalter 2002). A recent series of studies has addressed 59 individuals working with acrylonitrile within acceptable workplace exposure levels (3 ppm under German regulations) (Thier *et al.*, 2001). The role of polymorphic xenobiotic metabolism in the response to acrylonitrile was addressed by genotyping for glutathione-s-transferase-theta-1 (GSTT1), -mu-3 (GSTM3), -pi-1 (GSTP1) and CYP2E1. Individuals with the Val^{104} variant of GSTP1 were found to have an increased level of N-(cyanoethyl) valine adducts compared with those who had two Ile^{104} (wild type) alleles. This was thought to be due to altered affinity of the enzyme for electrophilic substrates. Further studies on the same individuals examined the role of CYP2E1 in acrylonitrile toxicity (Thier *et al.*, 2002). It is known that humans have a higher level of CYP2E1-mediated oxidative metabolism of acrylonitrile than rodents, suggesting that humans may have increased susceptibility to acrylonitrile toxicity compared with rodents. By using PCR-RFLP (polymerase chain reaction – restriction fragment length polymorphism) to look for 7 polymorphic variants of CYP2E1 as well as measuring levels of N-(cyanoethyl)valine in relation to acrylonitrile exposure, the authors identified a trend towards higher adduct levels in individuals with at least one copy of the A316G variant of CYP2E1, which is associated with lower levels of oxidative metabolism. The higher adduct level detected in these individuals may reflect the fact that more of the acrylonitrile is going via the reductive pathway and hence that these individuals are protected against acrylonitrile toxicity. Although this study was too small to be conclusive, the combined results of the studies discussed above suggest that individuals carrying the Val^{104} variant of GSTP1 along with the A to G^{316} variant of CYP2E1 may be at reduced risk from the acute toxicity of acrylonitrile.

Environmental exposure.

5.49 Polymorphisms may affect susceptibility to ingested environmental exposures due to altered uptake or clearance of toxicants from air, drinking water or food.

Recreational drugs

5.50 Polymorphisms in important enzyme systems may affect susceptibility to the adverse effects of common lifestyle factors such as alcohol consumption. Two enzymes, alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) are involved in susceptibility to the toxic and addictive effects of alcohol consumption. Individuals who carry the ALDH2*2 allele, which encodes an inactive form of ALDH, develop high peripheral blood acetaldehyde concentrations following alcohol consumption. The accumulation of acetaldehyde in the blood induces unpleasant effects that encourage the individual to consume less alcohol than average, and they are therefore less likely to abuse alcohol or become alcoholics. The activity of ALDH in the human brain is reduced in chronic exposure to alcohol causing increased acetaldehyde concentrations in the brain upon exposure to alcohol (likely to be more severe in individuals with the ALDH2*2 allele). It has been postulated that acetaldehyde and alcohol might act synergistically affecting some of the the multiple neuropharmacological and behavioural effects of ethanol in chronic alcohol abusers, with acetaldehyde reinforcing the hypnotic and amnesic effects

(Quertemont 2004; Quertemont and Tambour 2004). Interestingly, Mexican American populations (which have an increased statistical risk of alcoholism) have an extremely low allele frequency of the ALDH2*2 allele, in addition to a low frequency of ADH1B*2 and a high frequency of ADH1C*2 and CYP2E1c2 alleles (Konishi *et al.*, 2004).

5.51 Attempts have been made to use information of this kind to model the complex hereditary control of ADH, taking into account polygenic control and polymorphisms at two allelic sites. Development of a PBPK model (Sultatos *et al.*, 2004) has indicated that, while peak blood levels and time to peak are only marginally affected by ADH genotype, the AUC of the ethanol blood decay curve was very sensitive to genotype. The predicted AUC for any genotype including the ADH1B*2 (high activity) allele was much smaller than those for any genotype without this allele. The simulations undertaken showed that individual variability in alcohol disposition is affected by ADH and that the degree of variability predicted was a function of dose.

5.52 Furthermore, chronic alcohol consumption is associated with an increased risk of upper aerodigestive tract (e.g. oesophageal) cancer. This appears to be due to acetaldehyde generated when ethanol is metabolised by alcohol dehydrogenase (ADH), and heavy drinkers who are homozygous for the ADH1C*1 (high activity) allele are predisposed to develop upper aerodigestive tract cancer, possibly due to elevated salivary acetaldehyde levels following alcohol consumption (Visapaa *et al.*, 2004).

Polymorphic xenobiotic transporters

5.53 Xenobiotic transporters play a key role in toxicokinetics because they influence the oral bioavailability, distribution and excretion of xenobiotics (Fromm 2004). The recent discovery of the importance of transporters, membrane proteins which mediate the cellular uptake and efflux of molecules, in controlling chemical transfer across lipid membranes is leading to an increasing awareness of the potential for interactions at such sites. As an example, a number of compounds can inhibit P-glycoprotein, leading to an accumulation of ligands for this efflux transporter in certain cell types. As research in this area progresses, the importance and extent of this type of interaction will become more apparent.

5.54 There is a marked degree of interindividual variation in the expression of the xenobiotic transporter protein P-glycoprotein (MDR1) in the small intestine and liver, and furthermore there is polymorphic variation in the sequence of the MDR1 gene. By the beginning of 2004, 29 SNPs had been identified in the MDR1 gene (Marzolini *et al.*, 2004a). A silent mutation (C3435T) is thought to be associated with low levels of protein expression, while two variants (G2677T/A) result in alterations to the amino acid sequence of the gene (Ala-Ser and Ala-Thr at amino acid 893). However, the functional significance of these amino acid variants is unclear.

5.55 The role of MDR1 polymorphisms in responsiveness to xenobiotics remains to be elucidated (Eichelbaum *et al.*, 2004), although there is some evidence that the oral bioavailability of digoxin is increased in individuals with the C3435T allele. It has been suggested that the 3435T allele is associated with susceptibility to Parkinson's disease in pesticide-exposed individuals, although this hypothesis is based upon two major assumptions: (i) that pesticides are substrates for MDR1 and (ii) that pesticide exposure does, indeed, cause Parkinson's disease (Drozdzik *et al.*, 2003). Meta-analysis of case control studies tends

to support the idea that pesticide exposure is associated with the development of Parkinson's disease, but this is by no means proven (Paolini *et al.*, 2004). Polymorphisms of the MDR1 gene are associated with marked species and strain differences in sensitivity to the toxicity of the avermectin group of veterinary drugs and pesticides (e.g. ivermectin). Species/strains affected include the CF-1 mouse, the collie dog and murray grey cattle.

5.56 The importance of the MRP2 transporter in xenobiotic handling is illustrated by the rare autosomal recessive disorder Dubin-Johnson syndrome, in which the biliary excretion of conjugated anions from Phase II metabolism is diminished leading to mild conjugated hyperbilirubinaemia and the formation of melanin-like deposits in the liver. This is too rare to be classified as a polymorphism, but a number of other SNPs have recently been identified in the MRP2 gene and their functional consequences are under investigation (Gerloff 2004).

5.57 Other drug transporters, including the organic anion transporting polypeptide (OATP) and organic cation transporter (OCT), have also been shown to be polymorphic (Gerloff 2004; Marzolini *et al.*, 2004b). OATP is localised in the liver, kidney, brain and intestine. Individuals with a variant form of OATP-C (OATP-C*15, $Asp^{130}Ala^{174}$) exhibit reduced total and non-renal clearance of the drug pravastatin compared with individuals homozygous for the OATP-C*1b ($Asp^{130}Val^{174}$) allele. The OATP-A transporter, which is also polymorphic, is localised in the capillary endothelial cells of the brain and may therefore play a role in the CNS limiting the uptake and toxicity of drugs and toxicants. Similarly, polymorphisms have been identified in the OCT1 transporter, which mediates hepatic uptake of cationic substrates. These may affect the hepatic excretion of cationic xenobiotics and metabolites, thus altering their hepatotoxicity.

Receptor polymorphisms affecting toxicokinetics

5.58 One polymorphic receptor whose effects on a specific toxic response have been studied in detail is the vitamin D receptor (VDR), which appears to affect the toxicokinetics of lead. The VDR polymorphism may be detected by a *Bsm*I RFLP; the two alleles are identified as B and b, and individuals are characterised as BB, Bb or bb. The BB genotype is found in 7 – 32% of Caucasians but only 1 – 3% of Asians, and this genotype is associated with a reduction in bone mineral density. The VDR is associated with calcium absorption through the gut and into calcium-rich tissues such as bone, and studies suggest that it may affect lead handling at these sites as well (Onalaja and Claudio 2000).

5.59 The VDR genotype of an individual is characterised as BB, Bb or bb. Subjects who carry the VDR B allele (BB or Bb) have larger increases in tibial lead concentration with increasing age than bb homozygotes (Schwartz *et al.*, 2000a; Schwartz *et al.*, 2000b).

5.60 Lead also has effects on blood pressure in exposed individuals. In one study, battery workers with the VDR BB or Bb genotypes had higher mean blood lead levels than did bb homozygotes, and this was associated with higher systolic blood pressure when blood lead levels exceeded 40 μg/dL (Ye *et al.*, 2003). Lead workers with at least one B allele had systolic blood pressure 2.7 – 3.7 mm Hg higher than that of bb homozygotes. The B allele was also associated with clinical hypertension with an odds ratio of 2 (Lee *et al.*, 2001).

5.61 These epidemiological observations are interesting, but the mechanisms involved and their relation to risk are unclear. The functional significance of the BB allele is not obvious, since it is not a coding or splicing variant. One possibility is that this polymorphism itself has no functional significance but is in linkage disequilibrium with an as yet unidentified "true" risk allele.

Sex

5.62 Sex-dependent differences in metabolism have been reported for some therapeutic drugs in humans but only minor sex-related differences in foreign compound metabolism have been reported (see below). More pronounced sex differences occur in rats than other laboratory species, and there are sexual dimorphisms in xenobiotic metabolism (Mugford and Kedderis, 1998). Female rats have 10-30% less total hepatic CYP enzymes compared with male rats, and female rats metabolise many drugs and compounds more slowly than do male rats. The levels of CYP3A in rats are dependent on the sex hormone environment of liver cells, with much higher activity in males than in females; the levels in males are reduced by castration and the activity in females is increased by the administration of testosterone. CYP3A enzymes are the major hepatic and intestinal forms of CYP in humans and are responsible for the metabolism of numerous therapeutic drugs and other xenobiotics; a sex-dependent difference is not seen in humans, although the database is inconsistent (Tanaka, 1999; Ghandi *et al.*, 2004).

5.63 Several members of the CYP2C sub-family are sex-specific in the rat, in contrast to the human forms of CYP2C (Blaisdell *et al.*, 1998). CYP2C11 (only expressed in male rats) is responsible for 2α- and 16α-hydroxylation of testosterone and progesterone, whereas CYP2C12 (only expressed in female rats) is involved in female-specific hepatic 15β-hydroxylation of C21 steroids (Doehmer *et al.*, 1993). This suggests that xenobiotics metabolised by CYP2C11 in male rats may be metabolised more slowly in female rats.

5.64 Differences in the metabolism of xenobiotics are less common in humans, but recent findings suggest that drug kinetics in women can differ from those in men. Where statistically significant sex differences have been reported for humans they are not usually more than 2-fold in magnitude, and the differences are reduced if the data are expressed on a body weight basis (Schwartz, 2003). Toxicokinetic differences between men and women may arise from differences in body weight and composition, plasma volume, gastric emptying rate, plasma protein levels, drug metabolizing activity, drug transporter function (Morris *et al.*, 2003), and excretion activity (Ghandi *et al.*, 2004).

Conclusions on Genetic Polymorphisms in Xenobiotic Metabolising Enzymes

5.65 The various re-sequencing programmes in the wake of completion of the human genome project have demonstrated that the human genome is highly polymorphic. On average, 1/1000 base pairs differs between any two individuals. These are known as single nucleotide polymorphisms (SNPs). The SNP consortia have now identified several million SNPs. Hence, the question is no longer whether a given gene is polymorphic, but rather what are the consequences of the various polymorphisms identified in the gene. Technology has now reached the stage where it is relatively easy to determine the genotype for multiple polymorphisms whereas establishing the functional consequence of a polymorphism can often be much more difficult. This has led to the application of SNPs in genetic association studies long before the functional implications of the polymorphisms are known. Whilst there are good reasons for doing this

in studies of genetics, there are less sound reasons for doing it in a study of gene-environment interactions in toxicology, where unfounded inferences are likely to be drawn.

5.66 More problematic is the increasing ability to genotype for many alleles at multiple loci. Hence, perhaps six CYPs, two NATs, three GSTs and various other enzymes such as sulphotransferases (SULTs) and UDPGTs, together with some of the transporters (e.g. MDR1 and MRP2) and regulatory proteins such as AhR and CAR, are regularly genotyped in such studies. Some of the polymorphisms will have known functional consequences but others will be equivocal, unknown, or even silent. Study design will often be of insufficient power to support all of the comparisons undertaken, with only a small number of each combination of genotypes per exposure group. The multiplicity of comparisons leads to an increased probability of a statistically significant result occurring by chance, particularly when the analysis giving such a result is a consequence of post-hoc considerations. In general, the literature on associations between genotype for XMEs and risk from environmental chemicals has usually resulted in low and inconsistent relative risks (d'Errico *et al.*, 1999).

5.67 There is no doubt that the ability to analyse populations for many polymorphisms will lead to greater insight into genetic factors responsible for susceptibility to various diseases. However, in considering the implications of genetic polymorphisms in risk assessment there are a number of points that need to be considered:

- Genetic heterogeneity does not represent a new form of variation within the population. It has always existed as part of the underlying differences between individuals. What is now becoming possible is to explain some of that variation in terms of known genetic polymorphisms.

- The extent of interindividual variability has been explored empirically on a number of occasions. Whilst not ideal (of necessity such studies have almost always been based on pharmaceuticals), these studies have established that the default uncertainty factor (see Chapter 3) is generally appropriate. Hence genetic variability must represent a component of this.

- A genetically-determined difference in metabolism does not necessarily translate into a similar difference in risk. Many other factors can contribute to variability and these are unlikely to all act in the same direction. This has the effect of dampening the net effect of the genotypic separation of groups on the basis of metabolic activity. Many factors can influence variability in the toxic response through an effect on the toxicokinetics, toxicodynamics, exposure patterns or subtle psychological factors. Sometimes one factor can dominate, but often random variability in other factors will tend to reduce the clear separation between sub-groups classified on the basis of a single variable. Thus genetic epidemiology studies to date have all indicated odds ratios of less than 2 for susceptibility to toxic agents, supporting the use of the present uncertainty factors.

- Phenotypic differences in the activity of XMEs between individuals are often characterised in well defined populations of subjects, focusing only on the pathway of interest. Whilst one can often demonstrate a 10-fold or greater difference between groups, this is frequently not reflected in a comparable difference in the kinetics of the parent, because of competing pathways. Hence, whilst there are some examples of striking differences in kinetics due to genetic factors, for the majority of compounds such is not the case.

- One application of such information on genetic differences is to replace a component of the uncertainty factor with a compound specific adjustment factor. Information on the consequences of genetic differences may be used to derive chemical specific adjustment factors. Human variability could be modelled by a distribution (usually log-normal) and a percentile of the distribution could be selected (ie. use of a completely probablistic approach) and used for the derivation of health-based guidelines. Before undertaking such an exercise it will be important to determine whether this is justified in view of probabilistic estimates of the proportion of the population protected by this approach. For example, applying the maximum separation for kinetics, on the basis of a genetic polymorphism, together with assuming a default (presumably) maximum difference for dynamics might indicate that a large proportion of the population would be inadequately protected. However, on a probabilistic basis, the chance of someone being at the extremes of susceptibility from both toxicokinetic and toxicodynamic perspectives or at opposite extremes of these perspectives Is small. None-the-less it is possible that some people might not be adequately protected by current approaches, and there is a need to consider how such variability should be characterised and dealt with in the risk assessment. It is also noteworthy that this problem applies not only to variability in kinetics and dynamics: it is a general problem that also relates to other parameters in which there is variability within the human population (eg. consumption data).

5.68 Research findings regarding the impact of genetic polymorphisms on susceptibility to the acute and chronic toxic effects of chemicals should be monitored systematically with a view to determining whether particular susceptible groups may be identified and protected, thus reducing uncertainty with regard to interindividual variation within the human population.

6 Sources of Variability in Toxicodynamics

6.1 Toxicodynamics can be defined as the process of interaction of chemical substances with target sites and the subsequent events leading to adverse effects (WHO, 1994). An important source of inter-individual variability is genetic, but variability can also result from physiological factors and environmental factors. Once a chemical has entered the body, it can interact with one or more components of the body, for example, enzymes, DNA or receptors, to produce a toxicological or pharmacological effect. An understanding of molecular mechanisms is a great aid to understanding toxicodynamics and the ability to evaluate relevant molecular targets from humans and animals in isolation is a major advantage of *in vitro* approaches. Many factors can influence how the body reacts to chemicals. Individuals may react differently to the same chemical in both quantitative terms, for example due to differences in sensitivity, or in qualitative terms due to anatomical and physiological differences, such as in males compared to females, or due to other inter-individual differences such as atopy (immune sensitivity). Large molecules such as peptides that target the immune system of one species may show a very marked inter-species variation, but this is not the case for chemicals in general.

Species Differences

6.2 The relevance of effects detected in animals to humans and species differences in sensitivity of the molecular target or response must be considered. When considering toxicodynamic variability, knowledge of the mechanism of toxicity (including the site of action and active chemical entity) can allow the risk assessment to take into account known differences in target organ susceptibility. The presence and activity of protective mechanisms such as glutathione conjugation can influence the sensitivity of the target to the active entity.

6.3 The relevance to humans of the mechanism of toxicity in a test species depends on the presence and importance of the critical target in human cells or tissues. The mechanism of toxicity for a particular effect may not be relevant to humans, allowing this effect to be discounted in the risk assessment. For example, d-limonene causes kidney tumours in male rats via a mechanism that involves α-2-u-globulin, which is specific to the male rat and is not found in female rats, which do not develop tumours, or in humans. Therefore the renal carcinogenicity of d-limonene in male rats is not relevant to humans (IARC 1999).

6.4 Sometimes it is not possible to discount completely an effect detected in animals that appears to have little relevance to humans, and the risk assessment should take account of this uncertainty. An example is the antioxidant food additive butylated hydroxyanisole (BHA, E320), which can induce forestomach tumours in rats. It might be assumed that this effect could be discounted because humans do not have a forestomach. However the morphology of the rat forestomach mucosa is similar to that of the lower part of the oesophagus in humans so that the possibility of effects in the oesophagus needs to be considered. Such consideration needs to take into account the fact that BHA is a nongenotoxic carcinogen and the pattern of exposure of the respective tissues in rats and humans will be very different (Kroes and Wester, 1986; COT 1992).

6.5 In most cases where there are clear differences in the *in vivo* response between animal species, it is not possible to determine whether the differences are due to toxicokinetics or toxicodynamics. In such cases the sensitivity of humans compared with animals species cannot be measured directly, although insights may be possible from observational epidemiology studies, or from studies in human volunteers using

sensitive, mild and reversible biomarkers of response (Renwick and Walton, 2001) or from *in vitro* studies. Recent work by the Institute of Life Sciences International (ILSI), the International Programme on Chemical Safety (IPCS) and others has led to the formalisation of the process of considering the qualitative and quantitative concordance between findings in experimental animals and in humans (Barton *et al.*, 2006; Carmicheal *et al.*, 2006; Cooper *et al.*, 2006; Doe *et al.*, 2006). This work is most advanced for carcinogens but is now being extended to non-cancer endpoints (Cohen *et al.*, 2004).

Physiological Factors

6.6 Physiological factors including age and sex can result in toxicodynamic variability in both humans and animals. An example of variability in toxicodynamics due to physiological factors is the difference between adult humans and children in the reaction to phenobarbital. In adults phenobarbital acts as a sedative, whereas in some children it induces a hyperactive response. It is possible that this paradoxical response to phenobarbital is due to differences between adults and children in receptor-drug interactions (Roberts, 1992).

Environmental, Social and Dietary Factors

6.7 The effects of environmental factors on toxicodynamics have been less well studied than those on toxicokinetics. In part, this is because it can often be very difficult to distinguish between effects on toxicodynamics and those on toxicokinetics. An exception is the effect of therapeutic drugs, and for this reason many of the examples, which show the potential for the environment to contribute to variability in toxicodynamics, relate to drug actions. By their nature, drugs are designed to produce a pharmacodynamic response and there are a number of examples of their interaction with the effects of other drugs. There are fewer examples of such effects involving other chemicals. The issue of interactions between different chemicals to give additive or synergistic effects on their combined toxicity has been considered in detail by the COT report "Risk Assessment of Mixtures of Pesticides and Similar Substances (COT, 2002b) and in the COT Annual Report for 2004 (COT, 2004c). A clear example is the effect of non-selective irreversible monoamine oxidase inhibitor antidepressant drugs on the response to tyramine, which is present in foodstuffs such as cheese, and which increases blood pressure if its metabolism is inhibited by the antidepressant.

6.8 Where the adverse effects of toxic substances are similar to those of physiological stressors, an interaction may be expected. Thus the additional metabolic demand within the auditory system generated by noise can enhance the auditory system damage produced by exposure to high doses of ototoxic antibiotics, carbon monoxide, or dinitrobenzene, even though the noise exposure may itself be below the threshold for producing hearing loss (Ray, 1997). Axons repairing after damage have greater physiological demands and are similarly more sensitive to neurotoxic chemicals than normal. Such physiological-toxicological interactions are analogous to the greater effect of disturbed calcium homeostasis during calcium deficiency (see section on polymorphic receptors), and similarly would only be expected to be significant when the physiological systems were stressed beyond their normal limits.

6.9 Nutritional status and other lifestyle factors can affect toxicodynamics. Changes in diet can cause increases and decreases in the prevalences of different types of tumours occurring in laboratory animals.

Variation in the results of different studies that is caused by dietary factors could be eliminated by standardising the diet used in toxicological studies. People habituated to opiate analgesics such as morphine have been known to tolerate up to 50 times the normal analgesic dose of that drug. This response is not due to a down-regulation of the opiate receptors or an increased metabolism of morphine. Instead the tolerance is due to an adaptive up-regulation of adenylate cyclase (produces cAMP from ATP) which counteracts the depression of this enzyme by the opiate receptors (Rang *et al.*, 1999). There is increasing evidence for receptor cross-talk within and between families. Hence, the aryl hydrocarbon receptor nuclear translocator (ARNT) competes for the aryl hydrocarbon receptor (Ah receptor or AhR) and hypoxia inducible factor alpha (HIFα). Ligands for the AhR may thus modify the response to compounds inducing hypoxia. Similarly, there is cross-talk between the oestrogen receptor (ER) and AhR.

Genetic Factors

6.10 Toxicodynamic variability due to genetic factors arises where there is more than one form of a target (receptor or enzyme) (i.e. polymorphisms – see above). For example depending on the genotype, two forms of the same receptor may be expressed by different individuals, with one form having a higher affinity for a toxic chemical. Individuals who express one form of the receptor may be more susceptible to the toxic chemical than individuals expressing the other form. An example of variability in receptors is that brought about by polymorphisms of the AhR (Wong *et al.*, 2001) (see below). An example of an enzyme that is important in target organ susceptibility to a toxicant and exhibits polymorphism is glucose-6-phosphate dehydrogenase, which protects haemoglobin in red blood cells from the oxidative damage caused by ozone, nitrogen dioxide, certain drugs and certain plants, notably broad beans. Individuals with low activity of this enzyme will be more susceptible to this oxidative damage (Amoruso *et al.*, 1986). There is a substantial literature on the role of polymorphisms in genes involved directly or indirectly in DNA repair in cancer susceptibility. However, the extent to which such genetic variability contributes to cancer risk from environmental chemicals is not known. A more recent example is that of abacavir, a nucleoside reverse transcriptase inhibitor used in the treatment of infection with human immunodeficiency virus (HIV). Subjects with the polymorphic variant HLA-B57 are much more likely to develop a hypersensitivity reaction after administration of this drug (reviewed in Hosford *et al.*, 2004)

Polymorphic receptors

6.11 Understanding of the role of receptor polymorphisms in determining susceptibility to toxic agents is much less complete than is the case with polymorphisms of xenobiotic metabolising enzymes, and the literature contains few solid data regarding the role of receptor polymorphisms in toxicity. There are a number of reasons for this; in particular, genotype-phenotype correlations are very difficult to determine for receptors because viable intact cell systems are required for analysis. However, a number of attempts has recently been made to address this area; the methods used to date include *in vitro* induction in human lymphocytes, recombinant systems and population studies. The following section attempts to summarise the current state of play, taking a "broad brush" approach and looking at the roles of receptors in a range of phenomena including susceptibility to adverse effects such as addiction as well as acute and chronic toxicity.

6.12 Polymorphic receptors are much less likely to be phenotypically expressed than polymorphic enzymes, as it is more likely that there will be compensatory mechanisms to bypass non-functioning enzymes.

The aryl hydrocarbon Receptor (AhR or Ah receptor)

6.13 One of the most important receptors involved in the response to toxicants such as polycyclic aromatic hydrocarbons and polychlorinated dibenzo-*p*-dioxins, polychlorinated dibenzofurans and PCBs is the *aryl hydrocarbon* receptor (AhR). This receptor mediates the pleiotropic response to planar aromatic compounds, including the induction of CYP1A1, and probably also the carcinogenic response to these compounds. It has been known for many years that the AhR in mice is polymorphic, and that the form in C57BL/6 mice is highly inducible whereas that in DBA/2 mice is resistant to induction. Polymorphisms have recently been identified in the coding region of the human AhR. These are clustered in exon 10, the transactivation domain, with polymorphism also existing in the 5' regulatory region of the gene (Harper *et al.*, 2002).

6.14 Investigation of 91 Caucasian individuals has revealed seven mutations in the AhR, although expression studies in peripheral lymphocytes did not indicate any significant differences in AhR expression levels between the different forms. It has been postulated that variants which markedly alter AhR expression levels could be incompatible with survival due to the role that AhR plays in cell cycle regulation and as the affected individuals would be killed such effects would not be detected (Racky *et al.*, 2004).

6.15 Studies in placental tissue have indicated that the affinity of the AhR for TCDD varies by at least 10 fold between individuals; however, this cannot be explained by reference to the polymorphisms which have been identified to date. Furthermore, *in vitro* induction of CYP1A1 exhibits a heterogeneous distribution; it is unclear whether this is due to polymorphisms in the AhR or CYP1A1 itself.

6.16 The effects of the AhR polymorphism on susceptibility to toxins is unclear; its significance with regard to chloracne, the best understood toxic response to dioxin in humans, has not yet been clarified (Harper *et al.*, 2002). Furthermore, despite intensive research over many years it is still unclear whether dioxin is actually carcinogenic to humans (COC, 2001), l*et al.*one what the role of the AhR in such an effect might be. Studies in China have failed to reveal any association between AhR polymorphisms and bladder cancer susceptibility, either in benzidine exposed workers or in individuals with no record of occupational exposure (Zhang *et al.*, 2002). Furthermore, epidemiological studies on AhR polymorphisms and lung cancer have indicated no clear effect with regard to either CYP1A1 inducibility, lung cancer incidence or histological type (Cauchi *et al.*, 2001). However, one rare combination of AhR variants (Lys^{554}/Ile^{570}) abrogates CYP1A1 induction altogether, and theoretically this may be associated with resistance to polycyclic aromatic hydrocarbon-induced carcinogenesis.

6.17 Further confusion is generated by the fact that the *Ah*-receptor nuclear translocase (ARNT) and the *Ah*-receptor repressor (AhRR) are also polymorphic (Scheel and Schrenk 2000; Harper *et al.*, 2002; Cauchi *et al.*, 2003). Polymorphisms in the ARNT2 gene have been evaluated in relation to orofacial clefting, but were not found to be associated with either cleft lip or cleft palate (Barrow *et al.*, 2002). One recent study identified four polymorphisms within the AhRR gene, but examination of 171 lung cancer patients and 164 controls did not indicate any key role of the AhRR in either CYP1A1 inducibility or lung cancer

susceptibility in the population tested (Cauchi *et al.*, 2003). However, in a reproductive toxicity setting the Pro-Ala mutation at amino acid residue 185 of the AhRR has been linked to the development of micropenis, possibly because it is associated with an increase in susceptibility to the undermasculinising effects of dioxin exposure *in utero* (Fujita *et al.*, 2002). This finding should be treated with caution, though, because the functional effect of the Pro-Ala alteration on the AhRR protein was not evaluated.

Hormone and neurotransmitter receptors

6.18 Although relatively little is known concerning the impact of receptor polymorphisms on toxic responses, some information about polymorphic receptors and xenobiotic responses can be gleaned from examining the pharmacology literature.

6.19 Polymorphisms have recently been identified in the androgen receptor (Oettel 2003). Three polymorphisms have been identified to date: G1733A, (GGC)*n* and (CAG)*n*. The last variant, which encodes a variable length polyglutamine repeat in the 5' end of the coding region of the first exon $(CAG)_{10\text{-}35}$, has been studied in some detail. In normal men the repeat length is 14-33; the length of the repeat sequence is inversely proportional to the transactivation activity of the androgen receptor and may be associated with androgen insensitivity syndromes. Interestingly, differences in repeat length do not seem to be associated with differences in serum testosterone levels. The consequences in terms of testosterone toxicity remain to be elucidated, but this polymorphism has implications in terms of both clinical androgen treatments and the possible consequences of lifestyle choices such as the abuse of anabolic steroids.

6.20 Adrenoceptors have been shown to exhibit a number of SNPs, some of which alter the signalling properties of the receptors or affect their rate of desensitisation (Lohse 2004). In the β1-adrenoreceptor there is a Ser-Gly polymorphism at amino acid 49 in the extracellular N-terminus and an Arg-Gly polymorphism at amino acid 389 in the fourth intracellular loop, which participates in G-protein coupling. In the β2 receptor there is a rare Thr-Ile polymorphism at amino acid 164 which reduces the efficiency of signalling and two polymorphisms in the N-terminus which affect agonist-induced down regulation. Finally, in the α2C adrenoceptor there is a deletion in the third intracellular loop which causes impaired signalling and seems to be associated with increased frequency/severity of heart failure. The role of adrenoceptor polymorphisms in xenobiotic responses remains to be clarified, although it has been shown that the Arg^{389} variant is more responsive to β-blockers than the Gly^{389} form. The chronic response to treatment of heart failure may also be better in individuals carrying the Arg^{389} variant.

Polymorphic receptors and neurological disease

6.21 Polymorphic receptors may play a role in the response to neurotoxicants. One important group of polymorphic receptors comprises the ligand-gated ion channels, which mediate the influx into cells of either cations or anions in response to excitatory or inhibitory stimuli. Excitatory signals are mediated by neurotransmitters such as acetylcholine, whereas inhibitory signals are mediated by glycine or γ-aminobutyric acid (GABA). In the case of acetylcholine, therefore, reduced receptor activity tends to lead to an attenuated response. Conversely, in the case of inhibitory receptors such as the glycine and GABA receptors, reduction in the activity of the receptor can lead to an excessive or exaggerated response to stimuli because the inhibitory effect of receptor activation has been lost. A good example of this is a

genetic disorder, hyperekplexia ("startle disease") in which sufferers exhibit an extreme response to alarming stimuli such as loud noises. This disease seems to be associated with mutations at codon 271 and 244 of the α1 subunit of the glycine receptor, and in one case with complete deletion of exons 1-6. Although genetic hyperekplexia is a familial disorder rather than a toxic response, it is of interest in the present context because its effects are very similar to those seen in cases of strychnine poisoning (Vafa and Schofield 1998).

6.22 Neurotransmitter receptor systems have been implicated in the risk of various addictive responses. In European Americans, for example, a series of markers in the middle and 3' end of the $GABA_A$ receptor gene has been linked to alcohol dependency (Covault *et al.*, 2004). Interestingly, a mutation in the $GABA_A$ receptor (β3 Arg-His at amino acid 192) has also been linked to insomnia, possibly mediated via decreased GABAergic inhibition leading to hyperactivity; however, the prevalence of this mutation is unknown so it may not fall within the category of a true polymorphism (incidence >1%) (Buhr *et al.*, 2002).

6.23 Polymorphisms in both the dopamine D4 receptor (120-bp VNTR polymorphism) and the catechol-O-methyltransferase enzyme (high activity Val^{158} variant) have been implicated in the risk of becoming a methamphetamine abuser (Li *et al.*, 2004). Furthermore, the dopamine D2 receptor, along with a polymorphic region linked to the serotonin transporter, may be a smoking-modulated genetic risk factor for alcoholism (at least in Mexican Americans) (Konishi *et al.*, 2004), although this receptor apparently does not affect susceptibility to opiate addiction (Gareeva *et al.*, 2002).

6.24 Smoking is a clear case in which an understanding of genetic factors related to addiction would be beneficial. However, early results of looking at obvious candidate genes have been disappointing. For example, four polymorphisms in the nicotinic acetylcholine receptor (CHRNB2) have been examined in non-smokers and in smokers classified as having low or high levels of nicotine dependence, but none of the polymorphisms tested was associated with smoking initiation or progression to nicotine dependence (Silverman *et al.*, 2000). The μ-opioid receptor gene (OPRM1) is an interesting example; only one gene encodes OPRM1, but differential splicing leads to the expression of functionally variant protein products. In addition, polymorphisms in the regulatory region of the gene increase the extent of interindividual variability in response to opioids (Chevlen, 2003), and relatively common coding region polymorphisms (A118G (10.5%) and C17T (6.6%)) may affect responsiveness to both the addictive and acute toxic effects of opioids (LaForge *et al.*, 2000a; LaForge *et al.*, 2000b). A recent case report examining side effects following morphine administration in patients with renal dysfunction compared one patient who was homozygous for the A118G variant of OPRM1 with one who was homozygous for the normal allele. The A118G homozygote tolerated morphine well despite a high plasma level of the active metabolite morphine-6-glucuronide, whereas the one homozygous for the normal allele did not tolerate the morphine well, experiencing severe sleepiness and drowsiness. This led the authors to suggest that the A118G allele is a protective factor in morphine toxicity (Lotsch *et al.*, 2002).

6.25 Another example in which a receptor polymorphism may affect susceptibility to the side effects of neuroactive drugs is that of the serotonin receptor, 5-HT(1B). This receptor has a variant, Phe-Cys at amino acid 124, which modifies its pharmacological properties. One of the consequences of this modification appears to be increased susceptibility to coronary vasoconstriction which occurs as a side-effect of sumatriptan therapy for migraine in a subset of patients with additional pathogenic factors such as coronary heart disease (Kiel *et al.*, 2000).

6.26 Recent studies have implicated the endocannabinoid system (a series of receptors in the brain and elsewhere in the body that modulates the effects of primary signalling molecules such as dopamine, GABA, noradrenaline and seratonin) with substance abuse, including tobacco smoking. There is evidence that polymorphisms of the CB1 subgroup of endocannabinoid receptors, involved in self-reinforcing behaviour, may contribute to interindividual differences in addiction and substance abuse (Zhang *et al.*, 2004).

Difficulties in making conclusions on polymorphisms of receptors

6.27 It will be obvious from the section above that our knowledge and understanding of the role of polymorphic receptors in interindividual variation in response to toxic agents, particularly in relation to the classical toxic effects of ingested chemicals, is in a very immature state at present. In many cases the comments above are based on very few publications, and sometimes only a single paper on a particular topic has been published. This makes a critical review of current knowledge very difficult at the present time. However, the literature in this area is expanding at an ever-accelerating pace and it is to be anticipated that soon it will be possible to draw valid conclusions in a number of areas which are, at present, extremely confused.

Identification of susceptibility factors

6.28 When considering risk assessment and risk management, it may be important to reflect upon whether reducing risk by limiting exposure is a benefit to a small number of high risk individuals or to the population as a whole. For instance, an individual gene may have small impact on individual susceptibility but a large impact at the population level because it is highly prevalent (reviewed in Brennan 2002). Thus, on a population level, more can often be achieved by making small changes which affect the majority of the population rather than dramatic changes which affect only a small high-risk group.

6.29 It is also important to note that polymorphisms affect the probability of a particular event but do not directly indicate outcomes; it may never be possible to predict precisely which individuals will be affected. In other words, identification of an individual with a "susceptibility gene" does not indicate whether or not a toxic response will occur in that particular individual; conversely, the absence of a susceptibility gene does not guarantee resistance to toxicity.

6.30 Many preliminary epidemiological studies aimed at the identification of risk factors in humans are small, and even follow-up studies may only have 150-300 cases and controls (Brennan, 2002). This number is sufficient to detect common polymorphisms which double risk, but will not detect rare polymorphisms or those that cause less than a doubling in risk. Much larger studies and/or meta-analyses are required to reveal small increases in risk, but these would be of limited importance for risk assessment as they would be covered by the usual uncertainty factor approach. It is also important to note that polymorphic variants may be present at different frequencies within ethnic groups; for example, 45% of Caucasians but only 10% of Japanese individuals are NAT2 slow acetylators. This may mean that the implications of the identification of a particular variant has different implications depending on the ethnic makeup of the exposed population. It may not always be possible to extrapolate risk assessments from one ethnic group to another (Renwick 1996).

7 Sources of Uncertainty in Hazard Characterisation

7.1 Uncertainty, sometimes called type B or epistemic uncertainty or reducible uncertainty, refers to lack of knowledge, which could be reduced by further investigation. Thus an uncertain quantity is one that has a true, but unknown, value.

7.2 Several types of uncertainty are recognised. Two examples are:

i. Parameter uncertainty refers to data values that are not known with precision due to measurement error or limited observations (sampling error). This may be due to random (chance) variation or there may be systematic error.

ii. Model uncertainty arises because models are used to convert experimental or epidemiological data into an estimate of risk, for example during extrapolation from animals to humans. The amount of uncertainty decreases the closer the model (experimental or epidemiological study) is to the population of interest (eg. UK human consumers). In general, the amount of uncertainty increases in the following order: human investigations, studies in primates, studies in rodents, *in vitro* studies. The uncertainty reflects the limited ability of mathematical models to represent the real world accurately and may also reflect lack of sufficient knowledge.

7.3 Uncertainty in hazard characterisation relates to the limitations of the available data, whether from animal studies, controlled human studies or epidemiological studies. With respect to toxicological studies, the uncertainty relates to lack of knowledge in areas such as:

- the quality of the toxicological data
- gaps in the toxicological database
- relevance of the endpoints of toxicity
- current state of knowledge

7.4 The quality and completeness of the data typically vary for different categories of chemical substance. For new chemical entities that are subject to regulatory review and premarketing authorisation there may be a complete package of recent toxicological studies conducted according to Good Laboratory Practice (GLP) standards and complying with recognised test guidelines, but there may be little or no human data. For older synthetic substances and environmental pollutants, the existing toxicological data are likely to be more variable, but there may be some human data, sometimes even human experimental data, that can be used in risk assessment. For many naturally-occurring compounds, the toxicological database is extremely limited.

7.5 Emerging technologies and scientific understanding may also lead to uncertainties, for example in relation to the relevance of new data for the risk assessment process. This emphasises the need for the risk assessment process to continue to evolve and for appropriate expertise to be included in expert advisory committees.

7.6 In some areas of science, sensitivity analysis has been used to describe which sources of uncertainty and variability have the greatest effects on the overall conclusion. Sensitivity analysis is not normally used in toxicology, where it is prudent to make conservative conclusions that ensure the safety of potentially exposed people. However, the COT has stated that physiologically-based pharmacokinetic or toxicokinetic (PBPK/PBTK) models could be valuable for performing sensitivity analysis as part of the risk assessment process (COT, 2003a).

Quality and Reproducibility of the Data

7.7 The guidelines of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) and the International Co-operation on Harmonisation Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) give advice on testing regimes for assessing the safety of human and veterinary medicines. The Organization for Economic Co-operation and Development (OECD) guidelines define the design, conduct and reporting of toxicity and toxicokinetic studies in a regulatory context. If such studies are conducted in accordance with Good Laboratory Practice (GLP), including quality assurance audit, then it is possible to judge the quality of the study and the accuracy of the reporting. Older regulatory studies may have been conducted in accordance with earlier guidelines, perhaps involving smaller groups of animals, less sensitive measures of toxic effect and not according to GLP. Similar problems also frequently apply to research studies reported in the scientific literature. In particular, GLP is generally not used in academic establishments, and studies not generated for regulatory purposes may be less rigorously controlled. There may be doubts about whether reported observations are treatment-related, or whether all possible effects were adequately investigated and reported. Furthermore features of the study important to regulators, such as purity of the test material, may not have been reported. Investigators may also have focussed on dose levels that are expected to produce effects, and therefore fail to identify a NOAEL, which is often needed for risk assessment purposes. Some of the reporting deficiencies may be beyond the author's control: well-controlled research published in renowned scientific journals will have been subject to editorial control, which may restrict the amount of methodological detail and negative data that can be included, and this may render interpretation of the results difficult. There have been occasional instances of scientific fraud in both regulatory studies and those published in the general scientific literature. GLP was a response to the former and is used in toxicological contract facilities, where it provides an audit trail, which makes falsification of data difficult. Measures are in place to prevent fraud in research facilities in a few countries e.g. USA (http://ori.dhhs.gov/), but there is no formal mechanism in the UK. Consideration of the reputation of the laboratory or researchers may sometimes be taken into account, but of necessity this will involve subjective judgements. However, overall limitations in the quality of study design and reporting frequently result in uncertainty with regard to the accuracy of reported observations. There is a systematic approach available for evaluating the quality of experimental toxicological data (Klimisch *et al.* 1997). The Klimisch Scale gives criteria for establishing reliability categories for toxicology studies, based on a consideration of the level of information detailed in the study/publication.

7.8 In some areas of toxicology (eg. rodent studies examining endocrine modulating effects – see Chapter 10) there have been problems in obtaining consistent reproducible results, even when the studies fit the

criteria for them to be regarded as being of good quality. There is a need for a pre-planned robust mechanism for assessing the results of studies that give contradictory results.

Uncertainties in the reference point on the dose-response relationship used for hazard characterisation

7.9 Although health-based guidance values are often set on the basis of a NOAEL, the NOAEL is not identical to the threshold of toxicity. There needs to be a certain amount of response before a difference between the responses of the control group and the test group can be identified. Modelling of the possible magnitude of response in toxicity studies has indicated that there can be about a 5% response at the NOAEL (Gaylor 1992; Leisenring and Ryan 1992; Allen *et al.*, 1994). Dose spacing adds further uncertainty as there is uncertainty regarding where the NOAEL lies on the curve in relation to the actual threshold of response. The dose taken as the NOAEL (ie. the highest tested dose level below the lowest tested dose to have an adverse effect) will be lower than the highest dose that would not cause an observable adverse effect if all possible doses had been tested. In addition, there is uncertainty about the significance of exposures less or greater than, but close to the NOAEL. The choice of the critical effect is crucial when determining the NOAEL. The approach makes the assumption that if a NOAEL that is protective against the critical effect is used to derive an acceptable exposure level, this will be protective against all effects. However, while this is reasonable, there is uncertainty about the implications of exceeding the ADI/TDI if effects have different dose-response profiles. The above procedure assumes that the NOAEL for the critical effect in humans will be greater than or equal to the critical NOAEL for experimental animals divided by the safety factor (usually 10) used to compensate for uncertainty about interspecies differences. Of course, if an adverse effect were to occur in humans at doses lower than this, humans may not be adequately protected. Then it would be necessary to identify the NOAEL for the critical effect in humans and to set the ADI/TDI on the basis of it.

7.10 Uncertainty is increased because of the differing practices of toxicologists in defining the NOAEL. The NOAEL is frequently considered to be the highest dose where no adverse effect occurs as defined by a pairwise statistical test between the test group(s) and the controls. There may be disagreement how to proceed if the dose group below that which shows a statistically significant pairwise difference from the controls shows a non-statistically significant difference. A p-value of 0.05 is usually regarded as the cut-off point for statistical significance (ie. $p<0.05$ is statistically significant).

7.11 The NOAEL depends critically on study design, the sensitivity of measurements of toxic endpoints, choice of doses, dose spacing and group size (statistical power) (Renwick and Walker, 1993). Thus two studies on the same chemical that are identical in every respect except the doses used can identify different NOAELs because dose-spacing is a major determinant of the NOAEL. The existence of a dose-response relationship increases the confidence in the NOAEL, although dose-response relationships are not fundamental for defining a NOAEL (in contrast to the benchmark dose level (BMDL), for which a dose-response is critical). Generally, dose response information from animal studies is limited as the OECD methodological guidelines for toxicological studies were designed to identify a NOAEL rather than a BMDL. (The benchmark dose (BMD) and the BMDL are discussed in more detail in Chapter 12). The studies often use only three test groups, of which one is designed to be a no effect level, one of which must show an effect and an intermediate dose which may or may not show any response. Thus, some 'dose response

curves' may consist of only two points. Study design can be greatly facilitated by knowledge of toxicokinetics and by the results of trial studies to determine suitable dose levels.

7.12 The NOAEL is itself the subject of statistical uncertainty and its reliability depends upon the power of the study. Confidence in the NOAEL could be increased by use of larger group sizes or smaller dose spacing (eg. by use of more groups at different dose levels). The recommendations given in methodological guidelines regarding the number of animals to be used per group and the number of groups to be used is necessarily a balance between maximising the power of the study (by using larger groups sizes or more groups) and ethical considerations that require the number of animals used in testing to be minimised. Experiments that use fewer animals tend to result in higher NOAELs associated with greater imprecision in the determined NOAEL (i.e. increase the chance of a false negative [type II error] at any particular dose level). Thus, a NOAEL derived using a small number of animals per dose group, results in additional uncertainty about whether the NOAEL is actually below the true threshold of response (see Brown and Erdreich, 1989). However, the selection of animal numbers per dose group is influenced by external drivers other than scientific ones regarding power in defining the NOAEL. Notably the use of 4 or 6 dogs of each sex per dose group (on animal welfare grounds), makes rigid adherence to formal statistical analysis inappropriate. Often, it is necessary to consider either trends, or the responses in individual animals, against the spectrum of effects observed in the study in question and other studies, before drawing inferences. A further problem is that the multiple endpoints studied in a typical toxicological study increase the possibility of type I errors (false positives). Yet another question raised by the concept of the NOAEL is what constitutes an adverse effect. For example with organophosphate anticholinesterases, it is generally considered that inhibition of butyrylcholinesterase in plasma is not an adverse effect (although indicative of exposure), but that inhibition of acetylcholinestase is an adverse effect. This is because butyrylcholinesterase does not have an essential physiological function, whereas erythrocyte acetylcholinesterase is the same gene product as the acetylcholinesterase that is vital for normal brain, nerve and muscle function. An additional difficulty is agreeing what degree of acetylcholinesterase inhibition is considered adverse (FAO/WHO, 1999).

7.13 The LOAEL is subject to many of the statistical problems discussed above in relation to the NOAEL. As with the NOAEL, using a LOAEL means the information about the shape of the dose response curve is not used, although the steepness of the curve should be taken into account when selecting an additional uncertainty factor to account for using a LOAEL rather than a NOAEL. Thus, when extrapolating from a LOAEL to a NOAEL there is uncertainty about how close the resulting figure is to the actual threshold for an effect. As is discussed below, where a health-based guidance value, such as a TDI, is defined by a LOAEL, an extra uncertainty factor of up to 10, frequently 3, is often used.

7.14 The use of any additional uncertainty factor lower than 10 would depend on a detailed study of the dose-response for the critical effect, to estimate how close the LOAEL is likely to be to the NOAEL. For biochemical or haematological endpoints this may involve looking for consistency between variables likely to be disturbed together (eg. enzymes and plasma proteins in liver dysfunction), as well as the dose response. For histopathological endpoints a detailed consideration of the pathology report in terms of incidence and severity of changes would be necessary. If the incidence of this effect and the severity grading are both low, then the LOAEL is more likely to be close to the NOAEL. The report of the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC Technical Report 85) on how to

distinguish adverse from adaptive effects is particularly useful in this regard, as it outlines a soundly based and robust framework for decision making in this complex area of uncertainty.

Gaps in the Database

7.15 Adequate toxicology studies of appropriate duration are key to the toxicological evaluation of a compound. A complete database includes the studies referred to in Chapter 3. For many environmental pollutants and naturally occurring chemicals there is a lack of adequate data, particularly on carcinogenicity, reproductive toxicity, neurotoxicity and immunotoxicity: this is partly because there is no sponsor to remedy gaps in the database. Such gaps are uncertainties. Formal systematic reviews (see Chapter 12) can be used to test the completeness of the data available.

7.16 A review of studies conducted during development of new pharmaceutical agents indicated that chronic studies, with the exception of carcinogenicity studies, did not reveal any toxic effects that were not observed in subchronic studies (Lumley and Walker, 1986). Investigations of other databases (the German authorities, the company Bayer, and VICH looking at data on pesticides) found that the results of sub-chronic toxicity studies were not reliably predictive of the results of chronic toxicity studies. Therefore, it may not be reasonable to make precautionary assumptions about the relationship between the NOAEL for non-neoplastic lesions in subchronic and lifetime studies, i.e. to extrapolate from short term to long term exposure. Furthermore, additional endpoints of toxicity may be observed in studies conducted in pregnant or immature animals, or using specialised protocols. It is not justified to make assumptions on whether developmental effects, neurotoxicity or immunotoxicity will be observed at similar, lower, or higher doses to those causing other forms of toxicity in adult animals.

7.17 If key genotoxicity studies have not been conducted, then it may not be reasonable to assume a threshold since it would not be known whether the compound is an *in vivo* mutagen and hence a potential genotoxic carcinogen. The Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment (COM) advice is that for *in vivo* mutagens it is prudent to assume that there is no threshold for mutagenity (COM, 2000a). For such compounds the conclusion would be that exposure should be ALARP (see Chapter 3). The COM/COC consideration of 3-monochloropropanediol (3-MCPD) is an example of how further data can reduce uncertainty. In 1999, the COM considered that "the *in vitro* data were positive and the available *in vivo* data were inadequate to provide reassurance that the activity seen *in vitro* was not expressed *in vivo*. It would be prudent to assume that 3-MCPD was an *in vivo* mutagen." (COT, 1999). However, completion of adequate and reassuring *in vivo* genotoxicity studies, together with consideration of a possible hormonal mechanism of carcinogenicity, led to the conclusion that a threshold could be assumed (COM 2000b; COC, 2000) and a TDI was subsequently established (SCF, 2001).

Relevance of the Observations

7.18 There can be uncertainty about whether effects observed in animals are relevant to risk assessment in humans: conversely it may be impossible to reproduce certain human effects in animals: both are examples of model uncertainty. Animal toxicity studies can be conducted at very high dose levels, at least when a substance is relatively innocuous. This can lead to adverse effects resulting from disturbances of homeostatic and physiological processes, which are not relevant to effects that would occur at lower

exposure levels. Such effects include tumours induced by non-genotoxic mechanisms. Administration to rats of high dietary concentrations of poorly soluble carbohydrates is known to cause mineral imbalance that can lead to lesions in the caecum, kidneys, adrenal glands, etc. (FAO/WHO, 1983), which again are unlikely to be relevant to humans exposed to much lower doses. Nevertheless, animal data are assumed to be relevant unless there is good reason to think otherwise. Similar considerations of dose may apply to extrapolation of human data from poisonings or occupational exposure to exposure to the same chemical in food. Human toxicokinetic data, when available, are likely to have been generated at single low dose exposures, and there may be uncertainty with respect to their relevance to repeated exposure at higher doses.

7.19 Neurobehavioural studies have recently been introduced into regulatory toxicity studies. However, these tests are still undergoing development and there is relatively little experience of their interpretation. Although stress-related effects produced via adrenal activation can be modelled well in laboratory species, disturbance of higher cortical function can probably only be modelled in primates. At present however, we have been unable to find any examples of chemicals producing disturbance of higher cortical function (e.g. hallucinogens) that do not also produce adverse effects on lower levels of brain function that are detectable in non-primate animals (see also Chapter 9).

7.20 Endocrine modulation (sometimes referred to as endocrine disruption) is an example of an area where there is particular uncertainty with regards to the relevance of the results of animal studies to human health effects. In part, the uncertainty relates to the importance of physiological differences between laboratory rodents and humans. Endocrine modulation is discussed more fully in Chapter 10.

7.21 A number of syndromes that occur in humans cannot adequately be reproduced in animal models, and therefore cannot be predicted from the toxicological data currently generated in animals. These include, for example, food allergy and intolerance, nausea, headache and fatigue; there may be others yet unknown. For some of these symptoms, such as nausea, novel methods of assessment are being developed. Some indications may also be obtained from observations of lethargy and decreased food intake. Consumers with concerns about wide-ranging symptoms frequently allege that various "synthetic chemicals" are to blame. There are no reasons to expect that the human body would react more adversely to anthropogenic non-nutrient chemicals than to other non-nutrient compounds that occur naturally as food constituents or are produced during normal food processes such as cooking.

Studies *In Vitro*

7.22 To date, few *in vitro* tests have been validated for use in regulatory toxicology. Those currently validated are for genotoxicity, corrosivity and phototoxicity. However, it is anticipated that there will be a number of additions to this list over the next few years. Notwithstanding the status of regulatory acceptance, *in vitro* approaches are invaluable adjuncts to conventional *in vivo* toxicity testing and to the design and interpretation of observations in humans, including epidemiological studies. Often, *in vitro* approaches are the most suitable and reliable for defining the metabolism and other aspects of the kinetics of xenobiotic compounds in humans. This information can either help in interpreting the toxicological database or it might be used as the basis of chemical specific adjustment factors (see this paragraph and Chapter 12). *In vitro* models can be invaluable in exploring modes and mechanisms of action, either at the cellular or

molecular level. Often, this is the only means of comparing key events of a mode of action in experimental animals and humans. The information can be used to help interpret *in vivo* studies, to assess the relevance of experimental findings for human risk and possibly to derive chemical specific adjustment factors. Such studies can also help in the identification of susceptible sub-groups and aid in the quantification of their difference in susceptibility. It might be possible to investigate human CYP-polymorphisms using genetically engineered batteries of cell lines expressing variants of some human CYPs, but the results of such studies are difficult to interpret as they are often not predictive of *in vivo* effects. Finally, the results of *in vitro* studies are often a key source of information for populating toxicokinetic and toxicodynamic models, particularly those that are physiologically based. In addition, it is often possible to obtain an estimate of parameter variability from such studies. Nevertheless, there remain considerable uncertainties in regard to the extrapolation of studies *in vitro* to humans, so that, certainly at present, while clearly desirable, complete replacement of animal tests is not possible (see House of Lords, 2002).

8 Variability and Uncertainty of Data in Studies From Humans

8.1 Although the identification and characterisation of hazards is often based on data from experimental studies, human data may be available from volunteer studies, epidemiological studies, clinical trials or case-reports.

8.2 Human studies can be classified into two broad categories:

1. Experimental, including:
    a. Clinical trials
    b. Field trials
    c. Community intervention trials
    d. Controlled studies of exposed humans
2. Non-experimental or observational, including:
    a. Cross-sectional
    b. Cohort
    c. Case-control
    d. Ecological

8.3 The fundamental difference between the two groups is that in human experimental studies there is an attempt to reduce variation by extraneous factors and to ensure that those exposed are exposed to a known amount/dose because the investigator has assigned them to that exposure.

8.4 However, many of the data collected and analysed are common to both types of study and include:

- Demographic and other baseline data on individual participants
- Information on health outcomes
- Measurements of exposure and/or assessment of exposure for participants or groups of participants

Data on other factors that may influence the Health Outcome

8.5 In epidemiological studies, it may not be possible to judge whether systematic error has been introduced or whether confounding factors have been adequately taken into account, due to restricted reporting in the publications that do not necessarily reflect the quality of the actual study.

8.6 Most agencies have expressed a preference for using epidemiological data rather than toxicological data for risk assessment where possible. It is therefore important to consider the nature of variability and uncertainty within these studies. In a similar way to experimental studies, variability in human studies arises from true heterogeneity across people, places and time, and uncertainty in these studies arises from a lack of knowledge about factors that affect the observed results. Thus variability can affect the precision to which results can be generalised, whereas uncertainty can lead to inaccurate or biased estimates. It is important to recognise that both variability and uncertainty are unavoidable and are often understated in epidemiological studies. Both qualitative and quantitative exploration of the importance and impact of the sources of variability and uncertainty, although time consuming, can lead to greater understanding and improved decision making.

8.7 There is potential for variability and uncertainty to exist throughout the whole process of designing epidemiological studies, collection and analysis of data and in interpretation of results. The last is usually discussed in a qualitative way with regard to various sources of bias and whether these might have an impact on the conclusions that can be drawn from a particular set of results. Important sources of bias include:

i. Selection bias, in which the study group does not reflect the population to whom it is hoped to apply the results. This can be due to exclusion of certain subgroups, refusals, loss to follow-up, etc.

ii. Allocation bias in which, in clinical trials, for example, participants are not allocated randomly to different treatments. Non-random allocation does not necessarily directly give rise to bias but could do so indirectly by distributing confounding factors unevenly between groups.

iii. Information bias in which errors occur in the measurement or estimation of outcome, exposure and other data. This can impact on the magnitude and direction of the risk estimate and on the generalisability of the results. For example, if non-differential misclassification of exposure occurs then this has been shown to tend to attenuate the risk estimate towards the null value of no association between exposure and outcome. Information bias might be called measurement bias when it relates to numerical data rather than nominal data.

iv. Follow-up bias in which some of the participants are unable to be contacted or withdraw from a study, leading to incomplete evaluation. Follow-up bias may be regarded as a special case of selection bias.

v. Confounding is a problem when, within the study sample, the exposure of interest is associated with a factor which independently determines risk of the outcome. A factor can also be associated with an outcome because it lies between the exposure and the outcome in the causal chain or because it occurs as a consequence of the outcome. Furthermore, if the confounding factor has been satisfactorily measured then it may be possible to elimiate its effect, even if it does not have the same distribution in exposed and unexposed. Effect modification may affect the extent to which findings can be extrapolated or generalised to other populations.

vi. Analysis bias in which an inappropriate or limited analytical method has been applied to the data.

8.8 In addition to these potential biases, consideration of other potential limitations arising from the design of the study is also needed. For example, the size of the study i.e. the power, the adequacy of the period of observation to detect the health outcomes of interest and the sensitivity of case ascertainment are important issues to be evaluated.

8.9 Qualitative analysis of uncertainty and variability in epidemiological studies can be valuable in that a systematic identification and discussion of major assumptions and the nature and magnitude of potential errors can be carried out.

8.10 Statistical methodology provides tools for describing uncertainty and variability in a quantitative way. Often these are limited to the presentation of results from statistical tests and confidence intervals. They may focus on random error and statistical variation by quantifying the scope for error from random statistical variation. For example the natural variation between individuals in terms of their reaction to a given exposure, the inherent randomness in how a hazard is spread throughout a medium and in how individuals come into contact with the medium, and the variation caused by sampling.

8.11 Quantification of uncertainty involves ideas of chance, randomness, risk, hazard and unpredictability. Probabilistic approaches in which mathematical or statistical models are developed facilitate the incorporation of (i) uncertainty analyses to examine the variation or imprecision in the output due to input assumptions, and (ii) sensitivity analyses to identify those model parameters to which the output is most sensitive, i.e. evaluation of the size of changes in model output as a result of changes of known size in individual model inputs. Quantification of the uncertainty and/or variability in the output from a model can be expressed as summary statistics or using probability-density or cumulative-distribution functions.

8.12 Mathematical and statistical models are most often used in epidemiological studies to characterise exposure and to describe the dose-response relationships between exposure and adverse health outcomes. Uncertainty in these models can be classified into three broad categories: scenario uncertainty, parameter uncertainty and model uncertainty. Scenario uncertainty can arise, for example, when carrying out an exposure assessment, from failure to take into account all sources of a hazardous exposure or assuming that populations are homogeneous in exposure patterns when there may be significant variations in some subgroups. Parameter uncertainty may arise from measurement error (including random error and systematic bias), the use of generic or surrogate data, and random sampling error. Model uncertainty arises through gaps in scientific theory or misunderstanding about the nature of the relationships and oversimplified representations of reality. Scenario uncertainty, parameter uncertainty and model uncertainty are not totally distinct from one another. There is some overlap. For instance, if not all relevant sources or determinants of exposure are taken into account (scenario uncertainty), there is potential for error in the assessment of exposure (parameter uncertainty).

8.13 Both classical and Bayesian probabilistic approaches are used in epidemiological studies as well as for risk assessment. The latter have the advantage of being able to incorporate expert opinion and uncertainty about this. For example, in models of retrospective exposure assessment there may be differing opinions as to the technology used in the past and the likely magnitude of exposure experienced.

8.14 Bayesian approaches are also increasingly being developed in the field of meta-analysis. Systematic review and meta-analysis methods are well established in the area of clinical trials and involve the collation of the literature on a particular area of interest, assessment of the extent and quality of the studies found and provision of a compilation of results, often including quantitative estimation of risk from the combined studies. These methods facilitate transparency and reproducibility of the methodology and results, and ease of updating. They can identify gaps in the knowledge base and areas for future research. Quantitative meta-analyses can give greater statistical power than single studies and provide a framework for investigation of possible sources of heterogeneity between studies and biases such as publication and selective reporting bias. In spite of controversy over the opportunity for bias and other sources of heterogeneity compared with clinical trials, these techniques are being increasingly used in epidemiological research and a number of guidelines have been produced recently on the topic. They are also being used to combine toxicological studies.

8.15 Systematic review and meta-analysis methods also offer the potential to improve the transparency and reproducibility of the processes by which decisions are made, by expert committees, from large numbers of toxicological and epidemiological studies. Elicitation of expert opinion and inclusion of this in structured models would enhance the evaluation of the effect of lack of knowledge (individual or collective) about a parameter, and would foster transparency of decision making. It would be appropriate to consider application of this methodology to some examples of concern to a committee to explore and evaluated the utility of this approach for standard setting.

Molecular Epidemiology

8.16 The field of molecular epidemiology offers the opportunity to combine the scientific disciplines of epidemiology and molecular toxicology to investigate interactions between genetics factors and environmental and other factors in the cause of disease. In long-term studies of populations it has been shown that exposures to relatively high levels of a hazardous exposure over long periods do not affect all individuals equally and there is increasing evidence that genetic factors influence the susceptibility and resistance of an individual to disease. There has been increasing research into the multiple pathways involved in disease processes, e.g. to the process of carcinogenesis and the importance of interactions. These include simultaneous exposure to different causal and different protective factors, and host:environment interactions including metabolic polymorphisms. Tools that are being rapidly developed included genomics, proteomics, metabolomics etc, often allowing investigation of a vast range of gene functions in simple, easy to use screening systems. Analysis and interpretation of results from these techniques is currently an area of on-going debate reflecting the lack of understanding of the function of many of the genes routinely investigated. This introduces a whole new dimension to the elicitation and interpretation of sources of uncertainty and variability. Future epidemiological research will need to incorporate measurement of susceptibility to aid investigation of disease pathways and to detect gene-environment interactions. This will require collaborative teams of epidemiologists, toxicologists and specialists in exposure measurement and assessment.

Human Experimental Data

8.17 Human experimental data of interest to the toxicologist are of two main types. The first are studies of absorption, distribution, metabolism and excretion, including studies using radiolabelled test compound. Such studies are much more frequently carried out with drugs intended for use in humans than with food chemicals and in the UK administration of radiolabelled drugs at doses above a specified level of radioactivity require the assent of the Administration of Radioactive Substances Advisory Committee (ARSAC). A second type of study that is undertaken in humans is that of tolerability and this is discussed below.

8.18 Human tolerability studies have been carried out with a number of food chemicals, and the design of such studies is primarily to confirm tolerability of proposed intakes. In addition, human data might be available for substances (mainly pesticides and veterinary drugs) that have been tested for possible development as human drugs. Many toxicological endpoints cannot ethically be elicited in human studies, and human experimental studies are of greatest value when there is a good biomarker of effect that can be measured below the threshold for producing toxicity, such as cholinesterase inhibition for anticholinesterase insecticides. A substantial number of organophosphate and carbamate insecticides have JMPR ADIs and ARfDs based upon human studies (Thomson and Richardson, 2004). In most cases, an uncertainty factor of 10 has been used, as the additional factor of 10 for interspecies variation is unnecessary. Many of the considerations in design of human studies are the same as those in animal studies e.g. adequate power to detect effects. It is worth reflecting however that the more complicated and the longer the study; the greater is the likelihood of subjects withdrawing. This will have two effects; it will diminish the power of the study and increase the chances that the remaining subjects will not be representative.

8.19 In recent years, the procedures for human experimental studies have become increasingly structured, because of concern about past experimentation that may not have been truly voluntary. The Declaration of Helsinki of 1964 (WMA, 1997) set out ethical guidelines for *inter alia* non-clinical human studies. In the UK, guidelines were established by the Royal College of Physicians of London on research on healthy volunteers (RCP, 1986) and on ethical Committees (RCP, 1990). Important points are that a detailed protocol is required for the ethical committee, procedures must be justifiable in terms of the objectives of the study, informed consent is required and volunteers may be paid for the inconvenience but not so much that they would act against their better judgement (see review by Wilks and Minton, 2000).

8.20 Some countries, notably Germany and Austria, will not countenance the use of human volunteer studies on pesticides in risk assessment. Furthermore, there has been recent activity in this area both in the USA (eg the National Academy of Science review and the subsequent setting up a Human Studies Review Board by the EPA) and in Europe (eg the unacceptability of the use of human data in pesticide risk assessment by the European Commission). Human studies can be particularly useful for identifying NOAELs for the anticholinesterase activity of certain pesticides. If the investigation of this effect is limited to animal studies, there will be more uncertainty about the guidance values subsequently established for some pesticides.

8.21 Variability and uncertainty can affect the results of human studies in the same way as animal studies. A further consideration is that criteria for exclusion of volunteers are often drawn so tightly that it is difficult to be sure that supposedly adverse effects are truly test-material related.

Case Reports and Case Studies

8.22 Case reports of exposure and sometimes poisoning and case series (reports of more than one such case) are frequently included in toxicological dossiers. These are only of limited value in setting ADIs or ARfDs, but can nevertheless be helpful to the toxicologist. They can help to establish whether a particular pattern of exposure of humans (usually acute) produced similar effects to those seen after the same pattern of exposure in animals i.e. whether there are major qualitative differences in toxicity. With food chemicals the intakes are rarely known with any exactitude, so it is difficult to establish quantitative relationships.

9 Developmental Neurotoxicity

Introduction

9.1 An area of current concern to risk assessors is developmental neurotoxicity. This evolving area presents particular problems in hazard identification and characterisation. It is an example of model uncertainty in that there is doubt that animal data can adequately model development of the human nervous system especially its high functions. Further, developmental neurotoxicity is an area of concern particularly with respect to pesticide toxicology, because insecticides are known neurotoxins.

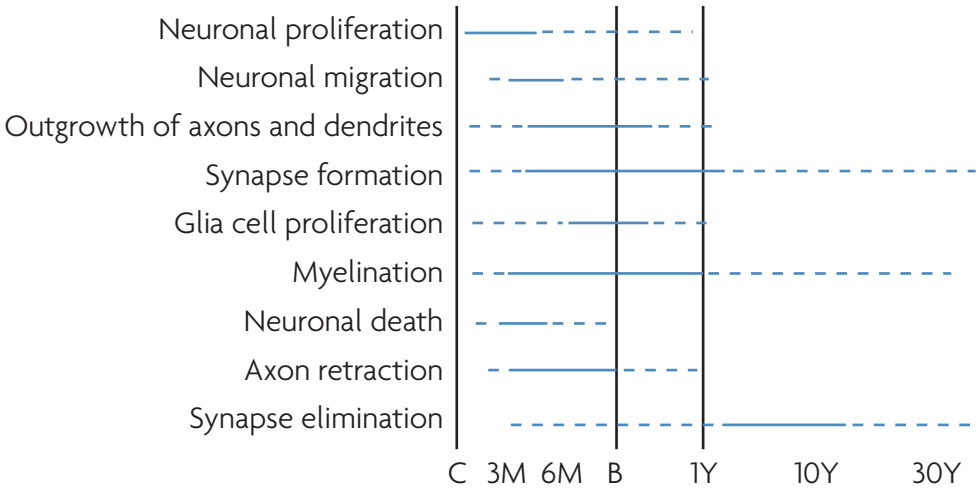
9.2 Developmental neurotoxicity in the human is a complex and poorly understood area. This is primarily due to the relative scarcity of information on the development of the human nervous system. It is known that the development of the central nervous system (CNS) is a long lasting and extremely complex process, but details on what exactly is happening are only currently emerging. The second major cause of the lack of knowledge in the field of human developmental neurotoxicology is the absence of well-controlled human studies (as the performance of such studies would be unethical) and the paucity of available epidemiological studies.

9.3 This Chapter discusses the consequences of the ontogenetic events for developmental neurotoxicity and presents examples of developmental neurotoxicological effects in the human.

Consequences of neuro-ontogeny for developmental neurotoxicity

9.4 The developmental processes in the telencephalon are summarised in Figure 9.1. It is clear that the development of the nervous system is a complex and long lasting process and that specific events occur during specific periods of life. The presence of time-windows with specific neuro-ontogenetic characteristics has two important consequences for developmental neurotoxicity.

Figure 9.1 Developmental processes in the telecephalon during human ontogeny



Schematic representation of the age of occurrence of various developmental processes during ontogeny of the human brain. A bold line indicates that the process mentioned on the left side is very active, a broken line that the process is active but to a lesser degree. Note that the age axis is drawn in arbitrary units.
C = conception, M = months, B = birth, Y = year.

9.5 Firstly, the presence of developmental periods characterised by specific neurodevelopmental events results in windows of specific vulnerability for adverse influences. This means that the effect of exposure to a specific event or substance depends on the developmental period during which the exposure occurs. A well known clinical example of the age-dependent effect of an adverse condition during early life is the difference in the effect of perinatal asphyxia in preterm and full-term infants. Perinatal asphyxia does not always result in brain damage. When it does, the lesion in preterm infants usually is localised in the periventricular regions, whereas in full-term infants the cortical areas, thalamus, basal ganglia and brainstem show a specific vulnerability (Volpe, 2001). Examples of the differential effect of age at exposure on outcome in the field of developmental neurotoxicity are the effects of prenatal exposure to radiation and alcohol. Follow-up studies on the effect of the atomic bombs in Hiroshima and Nagasaki revealed that the highest risk of mental retardation occurred in children who had been exposed to radiation *in utero* at between 10 and 17 weeks after the last menstrual period, i.e. during the period of abundant neuronal proliferation. Exposure prior to this period did not result in an increased risk of mental retardation and exposure during later phases of fetal life were associated with an only moderately elevated risk of low cognitive function (Otake & Schull 1984). Recent studies on the effects of prenatal alcohol exposure have indicated that alcohol exposure may interfere in particular with programmed cell death (apoptosis). This means that exposure to alcohol during the second trimester of pregnancy is especially likely to be associated with an adverse outcome (Olney, 2002).

9.6 Secondly, the age-dependent characteristics of the nervous system have consequences for the age at which evaluation should be carried out for adverse effects on its development. The continuous developmental changes in the nervous system are paired with continuous changes in function and, in case of dysfunction, in age related expression of dysfunction. Young infants can express dysfunction of the brain only by means of generalised and non-specific dysfunction. For instance, an infant with a left-sided intraventricular haemorrhage may respond with a generalised hypotonia, a generalised hypertonia, a hyperexcitability syndrome, or an abnormal quality of all body movements (Prechtl, 1977; Hadders-Algra, 2004). Infants cannot express dysfunctions in specific cognitive functions, such as attention deficits or problems in reading, spelling or mathematics. In general, these dysfunctions first emerge at school-age (Hadders-Algra, 2002). Other disorders are first expressed in early adulthood. A case in point is the development of schizophrenia: evidence is accumulating that this disorder might be related to adversity during prenatal life, such as malnutrition during the first trimester of pregnancy (Susser & Lin, 1992) or infection during the second trimester (Watson *et al.*, 1999). This means that the developmental sequelae of early exposure to a toxic substance may be first expressed after a latent or silent period of many years. On the other hand, the marked developmental changes of the brain may induce disappearance of toxicologically induced dysfunctions present in early childhood (Hadders-Algra, 2002). Thus, the age of assessment of potentially adverse effects of a toxic substance has a major effect on the outcome of the assessment, and this must be considered in the design of developmental neurotoxicity tests.

9.7 Plasticity is a characteristic of the nervous system that helps to reduce the effect of insult. There is a great deal of duplication of function in the immature nervous system, much of which is subsequently reduced by programmed neuronal death and synapse elimination during development. This means that limited and diffuse neuronal damage occuring prior to this elimination of duplicated functions may not have any adverse consequences if it is restricted to those elements that would have been removed anyway during normal development. Additionally there is some plasticity within the adult nervous system, and this can

mean that lost functions may be taken over by surviving neurones. A consequence of this is that the effects of a given developmental insult may be either reduced by favourable nutritional or educational/social factors, or increased by unfavourable ones that limit plasticity. Whilst plasticity has often been invoked to explain the resistance of the developing brain to mild generalised insult, insults specific to a particular brain area or function may disrupt the normal tightly integrated sequence of development beyond the capacity for correction by functional plasticity. It has also been shown that although plasticity may be sufficient to normalise unstressed function after neurodevelopmental insult, the response to challenge with pharmacological agents may still be abnormal - (based on data from animal studies) – see the section on "Developmental neurotoxicology tests" later in this Chapter (paragraph 9.30–9.32).

Food

9.8 In this section examples of developmental neurotoxicological effects of two types of chemicals in food will be presented: the effects of some specific food components and the effects of some environmental pollutants.

9.9 Currently, interest in the effect of early nutrition on the developing nervous system is growing. The interest has been inspired by the consistent finding that children who had been breast fed had an IQ advantage of 3 to 6 points when compared to children who had been bottle fed, an effect which remained after correction for confounders such as social class and parental smoking behaviour (Anderson *et al.*, 1999). The presence of long-chain polyunsaturated fatty acids (LC-PUFAs) in breast milk might be one of the factors underlying the developmental difference between breast fed and formula fed infants. Recent studies indicate that LC-PUFAs indeed may exert a temporary beneficial effect on neurodevelopmental outcome during early infancy (Simmer, 2001; Bouwstra *et al.*, 2003; Dijck-Brouwer *et al.*, 2005). Other studies suggested that low prenatal LCPUFA status and high transfatty acid status was negatively associated with neurological condition during early infancy (Dijck-Brouwer *et al.*, 2005, Bouwstra *et al.*, 2004).

9.10 A topic of current concern in child psychiatry is whether low LC-PUFA status plays a role in the development of attention deficit hyperactivity disorder (ADHD) and whether nutritional supplementation with LC-PUFAs can improve behaviour in children with ADHD. Until now no consistent evidence has been provided that this is the case (e.g. Voigt *et al.*, 2001).

9.11 A similarly topical issue is whether food colouring chemicals affect the developing nervous system. Here some evidence has been furnished that a minority of children may have a specific sensitivity for food colouring, but this was inconclusive and not confirmed (reveiwed in COT, 2000). Further investigation is underway (COT, 2002a).

Developmental Neurotoxicology in the Human

9.12 Each substance known to affect the function of the adult human nervous system has the potential to have long-term effects on the developing nervous system (Swaab *et al.*, 1988). This means that many medical and 'recreational' drugs have a neuroteratogenic potential. However, relatively little is known on the exact nature of neurotoxicological effects of specific substances during human development. Studies in humans

which investigate associations between exposure to potential neurotoxicants and neurobehavioural changes are hampered by the fact that they have to be controlled for many confounding factors such as social class, parental education, the child's gender, and parental smoking. The best studies are prospective in nature, which means that they have to follow individuals exposed during early life for many years. This is not an easy task in a mobile society. The studies available in general address the effects of prenatal exposure of a chemical or the exposure related to breast-feeding. Examples of neurotoxicological effects of various groups of chemical substances are discussed below.

Medical drugs

9.13 Antiepileptic drugs are the drugs of which the potential teratogenic effects have been studied most widely. During the 1970s it became evident that children who had been exposed prenatally to the antiepileptic drugs phenobarbital and phenytoin (often used together as combined therapy) showed a distinct pattern of physical abnormalities, including mid-face and digit hypoplasia, major malformations and growth retardation (Hill *et al.*, 1974). Gradually, the picture emerged that *in utero* exposure to antiepileptic drugs is associated also with an increased risk for a subtle degree of cognitive dysfunction (some points reduction in intelligence quotient (IQ) and an increased risk of attention problems; Van der Pol *et al.*, 1991, Gaily *et al.*, 2004). The risk appears to be associated with prenatal phenobarbital, phenytoin and valproic acid exposure, but not to exposure *in utero* to carbamazepine (Van der Pol *et al.*, 1991, Scolnik *et al.*, 1994, Gailey *et al.*, 2004). The risk increases when the fetus is exposed to multiple antiepileptic drugs.

9.14 Few studies have addressed the effect of prenatal exposure to benzodiazepines or to antidepressant drugs. There is no consistent evidence that these drugs have an adverse effect on neurodevelopmental outcome in childhood (McElhatton, 1994, Nulman *et al.*, 1997, Casper *et al.*, 2003). Prenatal exposure to the alpha-adrenergic antihypertensive drug clonidine may be related in a dose-dependent way to the development of sleep disturbances in childhood (Huisjes *et al.*, 1986). Exposure *in utero* to the anticoagulant coumarin, in particular exposure during the second and third trimester of gestation, appears to be related in a dose-dependent manner to the development of minor neurological dysfunction at the age of 7 to 15 years, but not to IQ deficit (Van Driel *et al.*, 2002).

9.15 Corticosteroids have been used in perinatal medicine since the 1970s, in particular in the form of two injections of corticosteroid into the mother before preterm delivery, as this treatment is associated with a reduction in respiratory distress syndrome, neonatal mortality and intraventricular haemorrhage. But evidence is gradually accumulating that repeated antenatal courses of steroids and high and/or prolonged dosages of postnatal corticosteroid in preterm infants is associated with an increased risk of neurodevelopmental disorder such as cerebral palsy. The risk may be higher for dexamethasone than for betamethasone (Whitlaw & Thoresen, 2000). In this respect the findings of the MRI study of Murphy *et al.* (2001) is noteworthy. They demonstrated that preterm infants treated with dexamethasone for neonatal chronic lung disease showed a 35% reduction of cerebral cortical grey matter at term age.

9.16 Since the 1980s serious birth defects have been reported after exposure during the first trimester of pregnancy to isotretinoin, an analogue of vitamin A. Retinoic acid is in general prescribed for severe recalcitrant cystic acne The abnormalities of retinoic acid embryopathy consist of malformations of the ear (microtia, anotia, absence or stricture of auditory canal, missing pinnae), CNS defects (microcephalus,

hydrocephalus, reduction deformities of the brain) and cardiovascular abnormalities (conotruncal defects, aortic arch abnormalities) (Lammer *et al.*, 1985).

Recreational drugs

Alcohol

9.17 Prenatal alcohol exposure can have serious and permanent adverse effects in children (Mattson *et al.*, 2001). The most serious outcome is the fetal alcohol syndrome (FAS), the diagnosis of which is based on three criteria: a) growth deficiency manifested by small overall height and small head size (microcephaly), b) central nervous system disorder, and c) a distinctive pattern of abnormal facial features. Other children with histories of heavy prenatal alcohol exposure and a neurodevelopmental disorder do not meet the diagnostic criteria of FAS. These children are labelled as having fetal alcohol effects (FAE). Children with FAS and FAE are born to women who drink heavily in an episodic fashion or more regularly drink substantial amounts of alcohol during pregnancy. These types of prenatal alcohol exposure are associated with cognitive dysfunction. Children with FAS and FAE show deficits in intelligence quotient IQ scores, which range from borderline scores (lower 70s) to average scores (90-109). IQs of children with FAS are more affected than those of children with FAE. Children with FAS and FAE in particular show deficits in executive functions, i.e. in a group of higher level cognitive abilities, such as problem solving and abstract thinking (Mattson *et al.*, 2001). In addition, it has been demonstrated that children with FAS and FAE are at high risk for behavioural disturbances, such as hyperactivity, impulsivity, delinquent behaviour, poor socialisation and communication (Roebuck *et al.*, 1999). The neuropathological correlate of these neurobehavioural dysfunctions are an overall reduced size of the brain, and a specific reduction in size of the hippocampus, the basal ganglia (in particular the caudate nucleus), the corpus callosum, and the anterior part of the cerebellum (Roebuck *et al.*, 1998).

Heroin and other illegal drugs

9.18 Studies indicate that prenatal exposure to heroin is associated with an increased risk of ADHD. It is also associated with mild impairments of cognitive function and impaired reading and arithmetic skills, but only when the offspring is raised by the heroin dependent mother. When the exposed children are adopted at young age they do not have an increased risk of intellectual impairment (Ornoy *et al.*, 2001). There is uncertainty about whether the effects are due to pre-natal neurotoxicological effects or environmental factors or a combination of the two. It is possible that later environmental factors might counteract the effects of *in utero* exposure.

9.19 Similarly there is uncertainty about possible interactions between antenatal drug exposure and environment in children prenatally exposed to cocaine (Brown *et al.*, 2004). Prenatal exposure to cocaine is associated in a dose-dependent way with deficits in mental, psychomotor and emotional development in early childhood. The effect in males is larger than that in females (Lewis *et al.*, 2004). The cocaine related effects may be abolished by the provision of non-parental, more optimal care (Brown *et al.*, 2004).

9.20 The developmental neurotoxicological effects of the recreational drug ecstasy (3,4-methylenedioxymethamphetamine) have not been investigated as yet. Prenatal exposure to ecstasy has been reported to be associated with an increased risk of congenital defects, in particular

cardiovascular anomalies and musculoskeletal anomalies (McElhatton *et al.*, 1999). Experiments in rodents suggest that prenatal exposure to ecstasy induces long-term changes in the monoaminergic systems, which are associated with increased locomotor activity in early adulthood (Koprich *et al.*, 2003).

Smoking

9.21 Maternal smoking during pregnancy is related in a dose-dependent manner to subtle global cognitive impairment, specific learning disorders and ADHD in childhood (Batstra *et al.*, 2003a; Linnet *et al.*, 2003). Remarkably, a recent study indicated that the adverse effect of smoking during pregnancy on the child's cognitive abilities can be eliminated by postnatal breast feeding (Batstra *et al.*, 2003b). The results of a study of 13 to 16 year-olds indicated that prenatal exposure to marihuana does not have an effect on later global IQ, but there is some evidence from one study that it does have a negative effect on tasks that required visual memory, analysis, and integration (Fried *et al.*, 2003). However, it is possible that this apparent effect might have been due to differences between the groups of marihuana users and non-users in societal factors such as family income, and proportion of married couples. A recent functional magnetic resonance imaging (fMRI) study indicated that these subtle cognitive deficits are mediated by altered functional activity of both frontal cortices and the right premotor cortex (Smith *et al.*, 2004).

Caffeine

9.22 The neurotoxicological effects of prenatal exposure to caffeine have been addressed in only one study (Barr & Streissguth 1991). The study did not find a negative effect of prenatal caffeine on parameters of IQ and attention at 7 years of age.

Contaminants that might be present in food

Mercury

9.23 An example of a dramatic neuroteratogenic effect of an environmental pollutant was the outbreak of Minamata disease in the 1960s in Minamata, Japan. Pregnant women had been eating fish highly contaminated with methylmercury. This resulted in a local large increase in the proportion of children who were born with severe developmental disabilities including cerebral palsy, mental retardation and seizures. It has not been entirely clear whether lower levels of prenatal mercury exposure are associated with more subtle degrees of cognitive and motor impairment (Davidson *et al.*, 2004; JECFA, 2003; COT, 2004a) (see Appendix 3). Methylmercury is discussed in detail as an example of risk assessment in Chapter 11 (paragraph 11.9) and in Appendix 3.

Lead

9.24 Lead is another heavy metal which is highly neurotoxic. Young children are especially prone to lead ingestion as they explore the lead-containing environment by means of hand-mouth behaviour. Many studies have consistently reported that blood lead level shows a strong negative correlation with IQ (Lidsky & Schneider, 2003; see also JECFA, 2000).

Polychlorinated biphenyls (PCBs)

9.25 Other environmental pollutants which may affect the development of the human brain are the PCBs and dioxins. Prenatal exposure to these substances is associated with a reduction in IQ and attention problems and subtle memory deficits in childhood (Patandin *et al.*, 1999; Jacobson & Jacobson 2004). The adverse effects of prenatal PCB exposure have been reported in particular in children who had not been breast-fed (Jacobson & Jacobson 2004). Follow-up of a cohort of perinatally exposed children at 6 years of age indicated that early PCB-exposure at current environmental background levels possibly induces transient delay in cognitive development rather than irreversible deficit (Winneke *et al.*, 2005).

Conclusions on developmental neurotoxicity in the human

9.26 It is notoriously hard to determine developmental neurotoxicological effects in the human. The source of the difficulty lies in the complex and protracted development of the brain, which is affected not only by specific chemicals but also by a multifactorial environment. However, this does not mean that developmental neurotoxicological studies are not possible. Animal data can offer insights into the mechanisms of possible developmental neurotoxicological effects and thus provide assistance in the design of studies in man. Human studies usually require a prospective design, controlling for many confounding factors.

9.27 At present, some medicinal drugs (e.g., phenobarbital, phenytoin, retinoic acid, dexamethasone, coumarin), some recreational drugs (e.g. alcohol, heroin, cocaine, cigarettes) and some environmental pollutants (e.g., mercury, lead, PCBs) are suspected of having a developmental neurotoxicological effect. Remarkably, some of the adverse neurotoxicological effects of prenatal exposure can be counterbalanced by environmental factors such as breast feeding or (in the case of drug-dependent parents) non-parental care.

Extrapolation from animals

9.28 Since most data for regulatory developmental neurotoxicity assessment comes from experimental studies in rodents it is necessary to consider the uncertainties involved in extrapolating from rats or mice to man. Although the same general principles of brain development apply to all mammals, rodents are born at a relatively immature state. Approximately 10 days postnatal in rats or mice corresponds to the degree of maturity reached around the time of birth in humans, which means that the rodent brain is deprived of the protection of the placental barrier to toxic chemicals at an earlier stage of development than is the case for man. However, not all brain systems develop in parallel, for example the rodent motor system develops relatively rapidly and the auditory and visual systems develop more slowly. Hence there is no age at which the whole rodent brain can be considered to be at a stage of development equivalent to the human. These differences in the timing of development can, to some extent, be compensated for in experimental studies by adjusting the timing of toxicant exposure to correspond with the approximate stage of development that is to be evaluated in man. However, since conception to weaning exposure is the norm for the toxicological testing of most substances (human pharmaceuticals being an exception), the timing of exposure is usually not a critical consideration and internationally-agreed methodological guidelines for developmental toxicity studies performed for regulatory purposes require the timing of

administration of the test material to cover the gestation period from implantation until the day prior to full-term of the pregnancy (OECD, 2001).

9.29 A more important factor that generates uncertainty in species extrapolation is the difficulty in finding measures for use in rodent developmental neurotoxicity screens that would be equivalent to some of the more complex disorders of human brain function, such as schizophrenia or autism, since these do not have exact equivalents in rodents. Although several rodent neurobehavioural tests have been developed that evaluate subtle functions sufficiently close to those disturbed in human schizophrenia to be of value in the development of therapeutic drugs (Wolf & Leander, 2003), these require training of experimental animals, and would involve a considerable increase in the time and financial cost of developmental neurotoxicity testing. In addition, some animal models of schizophrenia demonstrate absence of abnormal findings in early life (when regulatory developmental neurotoxicology assessment is carried out), with significant derangement of behaviours characteristic of schizophrenia appearing only in mid-life (Al Amin *et al.*, 2000; Howland *et al.*, 2004). The relatively simple tests currently employed (habituation of open field activity and Morris water maze learning) do not always detect subtle schizophrenia-like effects (Flagstad *et al.*, 2005), but would however detect a more widespread disturbance of brain development especially at the maximum tolerated dose (MTD) used in toxicological testing. Although several agents are known that can produce subtle disturbances of brain development in man and rodents, higher dose exposure to these same agents also produces more widespread effects on the brain that would be expected to be detectable by the measures currently used. The observation that the regulatory rodent developmental neurotoxicity tests carried out on pesticides to date have not set health based guidance values lower than those already in place for other end points provides some reassurance. However this observation could be interpreted as either a demonstration of the lack of neurotoxicity at low doses or of the insensitivity of the regulatory tests to disturbance of higher cognitive function. Better infomation comes from comparison of rodent test results with human health outcomes. The few toxic agents (as opposed to psychoactive drugs) that disturb higher brain function and have been evaluated both in man and in animals do provide reassurance that the current x10 interspecies factor provides adequate protection (see paragraph 11.9 and Appendix 3 on methylmercury) without any need for an additional factor specifically for developmental neurotoxicity. However, there are at present relatively few such examples, and the area needs to be kept under review. Equally, few chemicals other than pesticides have been subject to specific developmental neurotoxicity testing, and the reassurance that can be derived from standard reproductive and multi-generation toxicity testing is limited.

Developmental neurotoxicology tests

9.30 Conventional toxicological studies measure some aspects of behaviour such as ability to eat, drink and reproduce. A number of studies have been carried out in which developmental neurotoxicity has been evaluated by dosing the mother and measuring outcomes in the dams and offspring. The United States Environmental Protection Agency (USEPA) has requested such studies to a specific protocol to be undertaken with neuroactive pesticides, and, in 1991, the USEPA issued a standardised protocol for such studies, which has since been revised (USEPA, 1998). Such studies have also been undertaken in other situations, with compounds such as drugs used in human medicine, although to very variable protocols. In the studies they surveyed, Middaugh *et al.* (2003) found that developmental parameters were more sensitive than maternal parameters in 15% of the studies and offspring behavioural parameters were

affected by agents in the absence of maternal toxicity in only 3% of cases. These studies of developmental neurotoxicity are complicated and their practical aspects have been reviewed (Cory-Schlecta *et al.*, 2001). The key features are dosing of laboratory animals through pregnancy and the postnatal period, with direct dosing by gavage of the offspring during lactation. In-life endpoints such as sensory function (sensory thresholds or visual processing) and motor function (changes in gait or presence or absence of tremors or myoclonus) are investigated. Reflexes may test both motor and sensory function. Memory, learning and social and sexual behaviour may also be measured. The brains of offspring, depending on the guidelines used, have to be examined histopathologically at the 11th, 22nd or 60th day post-natally (Garman *et al.*, 2001) and morphometric analyses carried out. Inevitably regulatory studies of this type represent a compromise between measurement of all endpoints that can be measured, and what is practicable (see review by Mileson and Ferenc, 2001), and there remain numerous questions relating to the ideal experimental design. To date, nearly all of these studies have been done in the rat, and species other than rodents and rabbits differ more from humans in respect to placental morphology (Page, 1993), so that the rat is a logical choice of animal. Another contentious issue is direct dosing of the unweaned pups by gavage. Exposure of the human fetus depends on whether the xenobiotic crosses the placenta but exposure of unweaned babies may be from non-food sources, a fact which is used to justify direct dosing of the unweaned pups. Furthermore there are practical and logistical problems with such dosing and there is concern that direct oral dosing of very young pups might result in injury or stress causing effects that are unrelated to the chemical (Dorman *et al.*, 2001).

9.31 A corpus of data on the developmental neurotoxicity test in rats, conducted to the USEPA protocol, is being accumulated in the USA (Fenner-Crisp *et al.*, 2005; Makris, 2005). Some of the evaluations are already available and a draft retrospective analysis has been carried out (http://www.mindfully.org/Pesticide/Developmental-Neurotoxicity-EPA12nov98.htm). One conclusion from this is that developmental neurotoxicity of pesticides has not been seen at lower exposure levels than has adult neurotoxicity, and that in consequence developmental neurotoxicity testing has not identified a need to set lower exposure levels. In this respect the pesticides evaluated thus far have proved less hazardous than agents such as lead or methylmercury.

9.32 One point in relation to neurodevelopmental toxicity that is a matter of controversy is the distinction made between developmental neurotoxicity in the absence or presence of maternal toxicity. Thus if an adverse effect in the young is seen in a developmental neurotoxicity study in the absence of maternal toxicity it will attract an additional safety factor of x10 in the USA, under the Food Quality Protection Act (USA, 1996). However if the same adverse effect is seen in association with maternal toxicity, it does not usually attract this additional factor. This is because, when the effect is a consequence of maternal toxicity, protection of the mother against adverse effects, using the default 100-fold safety factor will also be protective of the fetus. However, it is important not to assume that developmental effects seen in the presence of maternal toxicity are consequences of such toxicity. This needs to be clearly established before concerns about the potential increased susceptibility of the fetus/newborn can be set aside. There is certainly evidence that maternal toxicity is very much a factor in human developmental toxicology (e.g. smoking).

10 Endocrine Modulation

Endocrine Modulators

10.1 As with developmental neurotoxicity, endocrine modulation is an area of current concern to the media and the public. This is an evolving area that presents particular problems in hazard identification and characterisation. It is an example of model uncertainty in that there are doubts about the general applicability of some of the animal data to the human, there are doubts about the usefulness of certain endpoints that have been used in animal models and there are doubts as to whether or not changes in some of these endpoints can be taken as evidence of an adverse effect. For example, studies which have investigated fetal exposure of rodents to estrogenic environmental chemicals may not be easily translatable to human health effects because of the very much higher levels of endogenous estrogens that occur during normal human pregnancy in comparison to rodents. However, even this comparison is not straightforward because in human pregnancy most of the circulating estrogen is bound to sex hormone-binding globulin (SHBG), whereas in rodents most of the circulating estrogen is in the free unbound form. In this regard, it is worth noting that much of the concern for potential adverse effects of estrogenic chemicals on reproductive development and health originates from experimental studies in laboratory animals, although studies in humans revealed the consequences of fetal exposure to diethylstilbestrol (DES). It is well established in the literature that exposure to high levels of DES results in adverse development of the reproductive system and cancers in both male and female offspring. However, DES is relatively unusual in that it does not bind to SHBG and therefore has availability in the human when compared with endogenous estradiol. Other issues such as species differences in absorption, metabolism and elimination of particular chemicals are also likely to occur depending on the nature of the compound and whether or not it binds to SHBG. Another aspect of the uncertainty relates to the endpoints, such as ventral prostate weight, uterine weight, which are used in the assessment of endocrine disruption potential in laboratory animal studies. It is uncertain whether a change in weight of these organs after exposure to a particular chemical indicates a truly adverse effect or one that may predispose to later disease. In addition to these uncertainties, there are other issues related to lack of reproducibility of results in different laboratories. This has particularly been the case for studies involving low dose effects of bisphenol A (Nagel *et al.* 1997), for which a satisfactory explanation has still not been made (discussed at paragraph 10.8). Possible explanations include differences in mouse strain, housing and dosing regimes. This issue has been the subject of considerable debate by expert groups around the world, most recently by EFSA (EFSA, 2006b).

10.2 Endocrine modulators (EMs) are compounds with the potential to alter hormone production, metabolism or action within the body. This is obviously a very broad and imprecise definition, as under its umbrella could be included not only various synthetic man-made chemicals but also natural components of the diet such as carbohydrates and sugars, ingestion of which alters hormone levels within the body. Endogenously produced hormones play vital and widespread functions in our everyday lives. Although blood levels of various hormones harmlessly fluctuate to some degree as part of the normal functioning of the body, it is well established that for nearly every hormone abnormally low or high production/action can result in disorders and sometimes even death (IPCS 2002). Unintentional exposure to an environmental chemical that is an EM is therefore potentially serious, assuming that the chemical in question is able to alter endogenous hormone production or action in a biologically meaningful way. The possible effects of plant EMs on health are discussed in the report "Phytoestrogens and Health" of the Committee on Toxicity of Chemicals in Food Consumer Products and the Environment (COT, 2003b).

Whether EMs do in fact impact significantly on human health, or whether this is merely a theoretical possibility, is a hotly debated topic in which various factions have expressed strong, opposing views (Sharpe and Irvine, 2004). It is a complex area and one that has been changing dynamically over the past few years as new information and understanding emerges. However, there are still many uncertainties and conflicting datasets, which make definitive conclusions difficult to draw (IPCS 2002).

Types of Endocrine Modulators

10.3 EMs can come in several guises. The first EMs to be identified, and the class in which more compounds fit than in any other, are those chemicals that have intrinsic hormonal activity i.e. they can bind to and activate the relevant hormone receptor; such compounds are hormonal agonists (IPCS 2002). The ability of chemicals to exhibit such activity can be detected *in vitro* using cell screening assays in which the cell in question expresses the relevant hormone receptor and some sort of response indicator.

10.4 The second class of compounds is similar to the first group in that the chemicals are able to bind to the relevant hormone receptor but in this case they are unable to activate its downstream signalling pathways. Instead, they occupy the receptor and block it from working; such compounds are classed as hormone antagonists (IPCS 2002). The majority of environmental chemicals that are classed as hormone antagonists are those that are able to bind to and block the androgen receptor (AR), examples being p'p'-DDE (the main and persistent metabolite of DDT) and vinclozolin (a fungicide) (Gray *et al.*, 2001). Although such compounds generally have some measurable antagonistic activity at lower concentrations than do EMs that are classed as hormonal agonists, it is a general principle that hormone antagonists have to be present at extraordinarily high concentrations to be able to block action of the relevant endogenous hormone.

10.5 The third class of EMs includes compounds that do not have any intrinsic hormonal or anti-hormonal activity but instead have the capacity to alter the production or metabolism of an endogenous hormone, and thereby to cause an endocrine change within the body (IPCS, 2002; Sharpe & Irvine, 2004). There are several major and important differences between this type of compound and the other two groups mentioned above. The most important difference is that such compounds cannot be detected by any simple *in vitro* screening system, as their EM-activity does not involve any intrinsic ability to bind to hormone receptors. Another key aspect of such compounds is that they pose a theoretically greater risk of causing a disorder, because they have the capacity to alter the action of endogenous, potent hormones (Sharpe and Irvine 2004); it is well established that when actions of endogenous hormones occur there can be health consequences. Another important consideration of such compounds is that because they alter endogenous hormones, their effect is difficult to distinguish from an abnormality in production or action of that particular hormone due to other effects in that individual, e.g. due to their lifestyle, genetic makeup or disease. There are a few examples of environmental chemicals, that possess more than one of the above three activities, or the compounds may be metabolised to several metabolites which individually may exhibit different EM activity. Examples include PCBs (IPCS 2002).

10.6 There are numerous uncertainties about EMs and it is these that have largely been responsible for fuelling both concern about their potential action and the polarised debate between environmental pressure groups on the one hand and the chemical manufacturing industries on the other. Screening programmes can be used to identify chemicals with intrinsic hormonal or anti-hormonal activity and this may then allow

some degree of risk assessment provided that there are data available on human exposure levels for that chemical; in practice, such information is very often lacking or is inadequate (IPCS 2002). Whether or not such EMs do pose a significant risk to humans can however be resolved by undertaking well-structured investigations that establish the level of human exposure and which then can enable estimation of risk based on measures of hormonal potency derived from *in vitro* and simple *in vivo* studies.

10.7 In contrast, identification of EMs that do not possess intrinsic hormonal or anti-hormonal activity, but which perturb endogenous hormones, is more problematical. Such compounds are unlikely to be identified by any simple screening system and therefore their identification tends to occur as a result of toxicological or other research investigations using particular classes of compounds in laboratory animal studies. A good example of how difficult it is to detect such compounds is illustrated by the fact that identification of the ability of certain phthalate esters to suppress testosterone production by the fetal testis (Mylchreest *et al.*, 1999; Parks *et al.*, 2000), was only discovered five years ago, despite the well-established testicular toxicity of these compounds and several decades of preceding in-depth investigation of the toxicological effects of this class of compound *in vitro* and *in vivo*. Establishing the potency of such compounds is also laborious as it depends on rigorous dose-response investigations *in vivo* using test animals rather than the use of *in vitro* cell screening systems.

'Low dose' effects of EMs

10.8 One of the most contentious issues regarding EMs is that surrounding the so-called low-dose effects of bisphenol-A in developing male rodents (i.e. effects including increased prostate weight in adulthood following *in utero* doses of 20 μg/kg or less). Such effects are disputed as not all studies have found them (IPCS 2002), and their relevance to human male reproductive health concerns is also unclear (Witorsch 2002; Sharpe 2003). Hormone levels can also be affected by lifestyle and dietary factors and it is difficult to distinguish between these effects and small effects of exogenous chemicals on hormone levels. What is certain is that, if the low-dose effects are real, they cannot result from the intrinsically weak estrogenicity of the compounds in question. Indeed, it is difficult to imagine that bisphenolic or other weakly estrogenic compounds can individually exert effects in humans as a result of their intrinsic estrogenicity at probable levels of exposure. This does not of course rule out the possibility that such compounds might exert effects as the result of some other biological activity, but other forms of toxicity have only been revealed at much higher dosages (in the mg/kg bw/day range).

Chemical mixtures and EM effects

10.9 Though the risk to human health of exposure to individual EMs probably does not pose insurmountable problems or raise any unsolvable uncertainties, this is not the case when considering the potential effects of combinations of environmental chemicals. In the real world, humans are exposed throughout their lives to a large number of environmental chemicals and it is reasonable to expect that it is the summation of effects of various EMs that might be important rather than the level of exposure to any individual compound (IPCS 2002). This is an intrinsically difficult area to investigate in a rigorous, scientific manner. To date only a few relevant studies have been undertaken, most of which have been *in vitro* (e.g. Rajapakse *et al.*, 2002) although there are a small number of relatively simple studies *in vivo* (Thorpe *et al.*, 2003; Tinwell and Ashby, 2004). The latter studies have been restricted to compounds which exhibit a similar

class of activity (estrogenic agonist activity). These studies have shown that when such compounds are combined at doses for which each individual component is below the level at which it will exert an effect itself, the combination of compounds together is then able to induce an effect. The effect appears to exhibit concentration additivity (see COT, 2002b), based on the small number of available studies.

10.10 An even greater problem is posed by studies designed to evaluate the safety of chemical mixtures in which the chemicals that are used exhibit different classes of activity e.g. an estrogen agonist plus an androgen antagonist or with a suppressor of endogenous testosterone production. In such instances it is probably impossible to interpret results from such studies as being additive, non-additive or interactive because the endpoint under investigation will not be a simple one that measures the action of an individual hormone but instead measures change in action of more than one hormone. This is not merely a theoretical issue but poses a genuine practical problem. For example, there have been several studies (McKinnell *et al.*, 2001; Rivas *et al.*, 2002) which indicate that alteration of the balance between androgen and estrogen action in developing male laboratory animals can result in disorders of the reproductive system, and in such instances it has been shown that it is the balance in action between the two hormones that is important, rather than the absolute levels of either hormone (Rivas *et al.*, 2002). As endocrine systems interact with each other, it is not difficult to imagine that effects of an EM in one system may have consequences for another system and that, via such effects, quite complex combined effects may emerge (IPCS 2002; Sharpe and Franks 2002).

10.11 The uncertainty in predicting the effects of mixtures of EMs is an example of toxicodynamic uncertainty, which is a major issue for future research. Mixtures of EMs also present problems for the regulation of chemicals. On the presumption that chemical mixtures are shown to have effects in toxicological studies at dose levels at which individual compounds do not have an effect, how will the individual compounds then be regulated? This is an issue which is yet to be addressed in any meaningful way but which is undoubtedly one that will become increasingly debated over the coming years. One possibility would be to base risk assessment on the defaults proposed in the COT report on Risk Assessment of Mixtures of Pesticides and Similar Substances (COT, 2002b), as agreed by COT in 2004 (COT, 2004c).

Age of exposure to EMs

10.12 The potential effects of EMs depend not only on their potency and the level of exposure, but also on the age at which exposure occurs and perhaps the duration of exposure. Of the latter two, age is by far the most important issue as far as EMs are concerned. The effects of EMs in adults are likely to be reversible upon cessation of exposure to the EM in question, whereas the exposure of the developing fetus to the same EM has the potential to induce an irreversible effect which may have lifelong consequences. In the fetus/neonate, the endocrine systems are still developing and their levels of negative feedback control (and other feedback systems), are in the process of being established and thresholds set. Inappropriately high or low exposure to a hormone at a sensitive stage, may result in abnormal setting up of the endocrine system and/or its feedback regulation. The consequence of such a change is that the endocrine system will function abnormally throughout life, this is therefore likely to lead to health consequences (IPCS, 2002). In contrast, exposure of an adult, in which the endocrine system is fully established and mature, will not result in any reprogramming change even though it may result in a period of altered hormone action. As mature endocrine systems are intrinsically homeostatic, they have the capacity to make adjustments for

altered hormone production or action that may result from exogenous exposures, at least when the effects of such exposures are mild (IPCS, 2002). It is primarily because of this difference in the potential effects in fetal and adult life that the risk from EMs is widely accepted as being a developmental issue rather than an issue for adults. There are however exceptions to this. For example, in the aetiology of breast cancer, it is well established that lifetime exposure to estrogens is of primary importance and therefore in this instance lifelong exposure to EMs with estrogenic activity is clearly an issue (Clamp *et al.*, 2002; The Endogenous Hormones & Breast Cancer Collaborative Group, 2002).

Human Data

10.13 Though there are a small number of good examples that indicate that human exposure to environmental chemicals, as a result of accidents resulting in locally high exposure, can cause health consequences (e.g. Dhara and Dhara, 2002; Pesatori *et al.*, 2003), there are no unequivocal examples that illustrate such effects resulting solely from exposure to an EM and working via an endocrine mechanism. This absence of clear, direct information is not necessarily conclusive as it can be extremely difficult in human studies to establish whether or not exposure to a particular chemical has exerted a significant effect (IPCS 2002). Such investigations are epidemiological in nature and are notoriously insensitive when it comes to relating chemical exposure to endpoint effects, the major stumbling block usually being the lack of good quantitative exposure data. Even more problematical is the situation when fetal exposure to an EM is thought to exert a health effect that does not manifest itself until adulthood, as in this instance cause and consequence may be separated by a period of 30 or more years (Sharpe and Franks, 2002). The two areas in which a potential role of EMs has been most debated and investigated has been in relation to breast cancer in women and to the occurrence of developmental disorders of the reproductive system in human males.

EMs and breast cancer

10.14 As a wide range of common environmental chemicals, both man-made substances and natural plant constituents, have been identified as having intrinsic estrogenic activity, it is logical that due consideration has been given as to whether human exposure to such compounds might contribute to life-time estrogen exposure and thus increase the risk of developing breast cancer. Most attention has been paid to bioaccumulative EMs that are lipophilic and thus accumulate with time in the fatty tissue of the breast where they may exert weak estrogenic action. The chemicals that exhibit such activities are primarily chlorinated compounds of one sort or another, such as organochlorine pesticides (DDT and related compounds) and other chlorinated compounds (e.g. PCBs). The first such study to be undertaken in a small number of women provided evidence of a link between increased exposure to chlorinated compounds and subsequent risk of breast cancer (Wolff *et al.*, 2000).

10.15 However, a number of studies since this original one, many of which were much larger and covered longer periods of time, have failed to confirm a significant or conclusive role for such compounds in the risk of developing breast cancer (Calle *et al.*, 2002). The COC has reviewed the possible association between organochlorine pesticides and breast cancer on a number of occasions and its most recent statement concluded that there was no convincing evidence that organochlorines are associated with the development of breast cancer (COT, 2004c). Though these findings are reassuring, they leave many uncertainties. For

example, the potential of mixtures of estrogenic EMs to exert significant effects is a real possibility based on the limited numbers of *in vitro* and laboratory animal studies that have been undertaken (Rajapakse *et al.*, 2002; Tinwell and Ashby, 2004). There are also concerns about whether exposure to estrogenic EMs early in life, particularly during fetal life, might have a 'reprogramming effect' which might fundamentally alter sensitivity of the breast to endogenous estrogens, thus increasing the risk of the individual subsequently developing breast cancer (Birnbaum and Fenton, 2003; Sharpe and Irvine, 2004).

10.16 Despite all the above uncertainties that relate to EM exposure and risk of breast cancer, it seems intrinsically unlikely that exposure to weakly estrogenic environmental EMs can play as important a role in determining the risk of breast cancer as do the potent endogenous estrogens that are produced by the woman herself throughout her reproductive life (Calle *et al.*, 2002). With this in mind, it is probably more logical to direct concern at exposure to EMs that do not have intrinsic estrogenic activity but which might have the ability to alter exposure to endogenous potent estrogens. Two such examples have been reported in the literature, namely PCBs and certain polyhalogenated aromatic hydrocarbon products of combustion. Both of these sets of compounds have been shown to be capable of suppressing the activity of endogenous estrogen sulfotransferase-1 (Kester *et al.*, 2000 & 2002), which normally sulphates estradiol prior to its excretion. Suppression of the activity of this enzyme would be expected to result in prolongation of the half life of estradiol and thus alter its potential to act on estrogen-sensitive tissues, such as the breast; such changes would occur without there being any change in the actual production of endogenous estradiol. However, it is unknown whether exposure to such compounds is sufficient to induce significant changes to estrogen exposure via such a route in the human.

10.17 There is also growing evidence that local production of estradiol within breast tissue may be an important factor in regulating estrogen exposure of the breast and thus of influencing risk of developing breast cancer (Suzuki *et al.*, 2002; Simpson 2004). Therefore, intake of EMs that have the ability to alter activity of aromatase {e.g. tributyl tin [TBT] (Cooke, 2002)} or estrogen sulfotransferases and/or sulfatases in breast tissue, may represent other routes via which estrogen action on the breast may be modulated locally (Zheng *et al.*, 2001; Coughtrie, 2002; Suzuki *et al.*, 2002). There are considerable logistic difficulties in distinguishing such effects from naturally occurring variation in estrogen levels between individual women, and at different phases of the menstrual cycle and at different phases of life. The potential for EMs that exert such effects to differentially affect individuals who may have differences in their ability to metabolise estrogens, for example due to a polymorphism in one or more genes encoding enzymes involved in estrogen metabolism (Coughtrie, 2002), is another area that merits investigation but which can only be speculated about at present.

EMs and Testicular Dysgenesis Syndrome

10.18 The second area of human health for which effects of EMs have been debated is testicular dysgenesis syndrome (TDS), which comprises various reproductive disorders, notably low sperm counts and testicular germ cell cancer in young adult men and incomplete testicular descent (cryptorchidism) and abnormal opening of the urethral meatus on the penis (hypospadias) (Skakkebaek *et al.*, 2001). The reason why EMs have been invoked as potentially playing a role in these disorders is not because of any conclusive direct evidence but because it is established that abnormal hormone production or action in fetal life is an important factor leading to development of all of these disorders. Suppression of testosterone

production or action and/or increased estrogen exposure during fetal life are risk factors for development of each of these disorders (Sharpe, 2003; Sharpe and Skakkebaek, 2003). Though humans are exposed to numerous EMs which have intrinsic anti-androgenic activity (e.g. DDE, vinclozolin) or those with estrogenic activity (e.g. bisphenolic and alkylphenolic compounds, chlorinated chemicals), it is probably unlikely that exposure of the fetus to such compounds is sufficient to disrupt normal androgen-dependent male reproductive development (Sharpe, 2003). This conclusion is based largely on studies in laboratory animals in which it has been shown that only exposure to very high doses of potent EMs with estrogenic or anti-androgenic activity is able to exert adverse effects on male reproductive development, and none of these has been shown to cause the combination of disorders comprising TDS (Toppari *et al.*, 1996; Sharpe, 2003; Sharpe and Skakkebaek, 2003).

10.19 In contrast, recent animal data has shown that exposure of rats during pregnancy to high levels of certain phthalate esters is able to induce in the male offspring a collection of disorders that is remarkably similar to TDS in humans (Mylchreest *et al.*, 1999; Fisher *et al.*, 2003), and this includes similar phenotypic changes to testicular structure and cellular development (Fisher *et al.*, 2003). Another change induced by phthalate exposure in fetal life in rats is a substantial reduction in anogenital distance, which is an androgen-dependent parameter, and which is normally approximately twice as large in males as in females. This is yet another example of a class of chemicals that do not have intrinsic hormonal or anti-hormonal activity but which are able to alter production of endogenous hormones, in this case the production of testosterone and insulin-like factor 3 by Leydig cells in the fetal testis. These two hormones are together responsible for testicular descent and for wider aspects of the masculinisation process. Human exposure to phthalate esters is widespread as such compounds are present in air, rainwater and in numerous materials and formulations to which humans are exposed on a daily basis. A number of recent studies have begun to document the level of human exposure to phthalate esters and these have indicated that women of reproductive age and children may be particularly highly exposed (Blount *et al.*, 2000; Koch, 2003; NHANES, 2003). Nevertheless, the levels of exposure still fall substantially below those which have been used extensively in the above-mentioned laboratory animal studies. However, a recent study in humans has produced preliminary evidence to suggest that phthalate exposure during pregnancy may be associated with a reduction in anogenital distance in boys (Swan *et al.*, 2005). This finding requires confirmation, but if it should prove to be true it would indicate that far lower levels of phthalates are able to exert 'anti-androgenic'effects in the human fetus than have been shown so far to occur in laboratory rats. There are however several uncertainties. For example, humans are exposed to a mixture of phthalates and therefore the summation of effects has to be taken into consideration; this poses some of the problems discussed earlier relating to the evaluation of mixture effects. Second, it is possible that exposure to phthalates may suppress endogenous testosterone and that such a change in combination with altered exposure to pregnancy estrogens (e.g. as a consequence of exposure to other EMs) might interact to increase the risk of developing TDS; interaction with other lifestyle effects or the genetic predisposition of the mother or fetus might also be important factors.

10.20 Aside from the data on phthalate esters, there are other pieces of information which more directly indicate that human exposure to environmental chemicals can result in permanent changes to the reproductive system/testis of the developing male with life-long consequences. First, two recent epidemiological studies have identified that male offspring born to woman who smoked during pregnancy have substantially smaller testes and lower sperm counts in adulthood than do the offspring

of mothers who did not smoke (Storgaard *et al.*, 2003; Jensen *et al.*, 2004). The mechanism underlying this effect is not established but clearly points to altered numbers of Sertoli cells in the developing testis. Recent evidence from laboratory animal studies indicates that lowered androgen action during this period in life can result in similar reduction in Sertoli cell number (Johnston *et al.*, 2004). Based on understanding of the interaction of some of the polycyclic aromatic hydrocarbons present in tobacco smoke with the aryl hydrocarbon (Ah) receptor provides at least a theoretical pathway via which suppression of androgen action in the fetal testis might be induced via an EM-like mechanism (e.g.. Kizu *et al.*, 2003). Second, there are two recent studies which reported higher levels of organochlorine chemicals, such as PCBs, in mothers of men with testicular cancer, the latter being one of the manifestations of TDS (Hardell *et al.*, 2003 & 2004).

10.21 With all of the examples for which possible involvement of EMs in human disorders are debated, there is great uncertainty about how to distinguish such effects from those that may be due to altered diet, lifestyle and/or social changes that have occurred dramatically during the last century or so (Sharpe and Irvine, 2004). These changes have undoubtedly had major impact on various aspects of human health and quite notably on endogenous endocrine systems. For example, the increased prevalence of obesity in Western societies will have quite dramatic consequences on the endogenous hormonal environment. Most obviously it affects various aspects of the hormonal systems that regulate glucose metabolism and fat deposition but these also have important interactions with sex steroids in a number of different ways (Haffner, 1996; Sharpe & Franks, 2002). For example, the aromatase enzyme is expressed in fat cells and it is well established that increasing obesity leads to increased expression of aromatase in such cells with consequent increase in capacity to produce estradiol from precursor androgens (Simpson, 2003). This will have implications for the risk of developing breast cancer. Similarly, such alterations in pregnant woman and associated changes in insulin resistance (due to obesity and consumption of a diet high in refined sugars) can lead to alteration in production of endogenous sex hormone-binding globulin (SHBG) to which both androgens and estrogens normally bind during circulation in the bloodstream. Alteration of SHBG production changes the bioavailability of androgens and estrogens and this may thus alter exposure of the fetus to these same hormones (Sharpe and Franks, 2002; Sharpe and Skakkebaek, 2003). However, this is largely theoretical speculation because of the considerable difficulties in establishing the extent to which maternal diet and lifestyle changes might affect hormone levels/exposure in the fetus, and it is even more problematical to imagine how such effects might be distinguished from the potential effects resulting from exposure to exogenous EMs.

Animal Data

10.22 There are several well-established examples in which exposure of certain wildlife species to environmental chemicals has been shown to result in health-related changes in individuals and populations as a result of disruption of one or more endocrine systems. Some of these might be directly relevant to man but in many instances they are probably not of direct relevance, for example those that involve exposures of aquatic animals via water. The most dramatic effects of environmental endocrine disrupters have been observed in aquatic systems. Though these are not be directly relevant to man in terms of the exposures involved, they provide examples of the mechanisms via which widespread adverse effects in vulnerable populations may be induced, and the lessons to be learned from these might be relevant to human concerns in this area. The two most clear-cut examples and those with the widest

prevalence are: 1) induction of imposex in certain marine snails, 2) the induction of intersex in male fish in various rivers in Europe and North America.

10.23 Imposex is a world-wide problem that results from exposure of susceptible species to organo-tin compounds, such as tributyl tin compounds, which are used as anti-fouling agents on ships (Evans & Nicholson, 2000; IPCS, 2002). Exposure to these compounds results in suppression of the aromatase enzyme in female snails with consequent build up of precursor androgens in these animals; this results in development of the vestigial penis which in turn results in mating and fertility problems. This is another example of an EM which has no intrinsic hormonal or anti-hormonal activity but which perturbs the normal endogenous hormonal environment (Sharpe & Irvine, 2004). Another recent study has shown that pregnant sheep that were reared on pasture fertilized using sewage sludge exhibited adverse effects on growth and testicular development in male fetuses. In this instance, there would have been exposure to a wide range of environmental chemicals that are present in the sewage sludge, and this could be viewed as to some extent mimicking the real world chemical environment in which we live. These examples are relevant to man in a general sense but the obvious uncertainties are whether or not humans are exposed to the same mixtures of chemicals and at comparable levels as occur in the various situations in which effects on wildlife or experimental animals have been demonstrated. In contrast, induction of intersex in male fish appears to result directly from exposure to EMs with intrinsic estrogenic activity. However, the main culprits in most instances appear not to be environmental EMs as such, but instead appear to be the potent estrogens, estradiol and ethinyl estradiol (used in the contraceptive pill or in hormone replacement therapy) that are excreted in the urine of women and which then find their way into river water via sewage effluent (Jobling *et al.*, 2002; IPCS, 2002). Though the exact mechanism via which this exogenous estrogen exposure results in intersex in fish is still unclear, it appears to involve a developmental mechanism and in certain general respects the resulting changes to the testis and reproductive tract in affected males have similarities to TDS in humans, as discussed above. Such parallels have added to the debate and concern about EMs and have helped fuel speculation about whether similar EM exposure in the human might play a role in TDS. However, though the human fetus also inhabits an aquatic environment, the level of EMs in this environment is determined by the level of exposure of the mother, rather than by the ambient EM burden. Nevertheless, it is intriguing to consider that the human fetus may be exposed to the same potent (pregnancy) estrogens in its aquatic environment as are fish living in river water in which sewage effluent is discharged. The extent to which the human fetus is actually exposed to pregnancy estrogens, and how this might vary depending on lifestyle or chemical exposures of the mother (e.g. to EMs that affect estrogen metabolising enzymes) is unknown, but is potentially important (Sharpe and Skakkebaek, 2003; Sharpe & Irvine, 2004).

10.24 There are a number of other examples in which disorders of reproductive/sexual development and function in wildlife have been shown to occur with clear evidence of relationship to exposure to environmental chemicals and/or EMs. Examples are alligators in Lake Apopka (Guillette and Gunderson, 2001), and a variety of changes in birds and in top predator animals such as polar bears (IPCS, 2002; Skaare *et al.*, 2002; Oskam *et al.*, 2003). However, in many of these instances it is not clear whether individual chemicals are responsible for the observed effects or whether it is a combination of exposure to mixtures of chemicals that is responsible (IPCS, 2002). It might be argued that evaluation of top predators such as the polar bear would be a wildlife animal model that has most parallels with the human.

In Vitro Data

10.25 Large amounts of data are routinely generated comparing the effects of chemicals on binding to the estrogen receptors, transcriptional activation of estrogen related genes and proliferation of cells, mediated through ligand binding to their estrogen receptors, *in vitro*. However, no risk assessment process has been developed that allows those data to be used for human exposure to estrogen-like chemicals.

10.26 A hygiene-based margin of safety (HBMOS) model for xeno-oestrogens exposure has been proposed for comparing chemicals with estrogen-like activity, mediated via the estrogen receptor, taking into account their potency and comparing this to the levels of estogen-like potency for well establishes dietary intakes of phytoestrogens (the isoflavones genestein and daidzein), considered to be acceptable for human consumption (Bolt *et al.*, 2001).

10.27 This approach has been used to benchmark the potential endocrine modulating effects of chemicals with very weakly estrogenic activity, such as the parabenzoic acid esters, against the potent endocrine disruptors diethylstilbestrol, and 17β-estradiol. This confirms the safety of human exposure to parabenzoic acid esters (Golding *et al.*, 2005).

10.28 In terms of estimating the increased exposure of humans to estrogen-like food ingredients, the cumulative exposure and potency of estrogen-like substances has been modelled for exposure to isoflavones in fortified food (Safford *et al.*, 2003). This exposure modelling is unique in that it considers both sexes and vulnerable subgroups within the population, based on their endogenous hormonal status, which differs considerably. It allows a consideration of the influence of additional estradiol-like chemicals/ingredients on the total estrogen body load for sub-populations using sensitivity analysis. Using this approach the relative variability in exposure measurements, and hence the uncertainty in likely biological effects, due to exposure, can be estimated. However, there is growing evidence that the effect of phytoestrogens *in vivo* is dependent very much on the endogenous hormonal environment, such that in certain situations the phytoestrogens act as estrogen antagonists rather than agonists. There is presently uncertainty as to what factors precisely determine whether or not the phytoestrogens will exhibit agonistic or antagonistic properties and until our understanding is improved, this will mean that attempts to model such effects will have their limitations.

Conclusions on Endocrine Modulation

- It is recognised that alterations in endogenous estrogen and/or androgen exposure play pivotal roles in the aetiology of breast cancer and TDS disorders in humans.

- If environmental chemicals are able to alter estrogen or androgen exposure in humans, there is the possibility that they may alter risk of developing these disorders.

- Based on current understanding, and the comparative hormonal potency of EMs and endogenous androgens and estrogens, it seems most likely that factors which alter endogenous hormone production or metabolism, and thereby hormone action, are intrinsically more likely to pose a health risk to humans. Such effects are perhaps more likely to result from changing lifestyle than from exposure to EMs, but distinguishing one from the other is extremely difficult.

- If EMs do exert effects in humans, they are most likely to occur in fetal or perinatal life and such effects would be likely to have life-long consequences.

11 Adequacy of Current Approaches

Introduction

11.1 Since the objective of regulatory toxicology is to avoid harmful exposures, it is difficult to investigate the adequacy of the procedures used, because if the risk assessment is correct, it will successfully prevent the adverse effects of interest. An "absence" of effects is thus necessarily difficult to evaluate and by the same token it is difficult to establish if the assessment was over-cautious. For chemicals that are present in food or the environment, it would be less hard to detect insufficiently cautious assessments, but the difficulty in detecting adverse outcomes and retrospectively relating any suspected events to exposure or dose means that this is not always possible. Similarly a small increase in the incidence of a particular event might go unremarked or be dismissed as an intercurrent illness unless there was clear evidence of recent exposure to a particular chemical. Association between chronic and/or delayed illness and exposure would be less likely be suspected than if exposure was acute and the effects seems shortly thereafter. Consequently, opportunities for the validation of toxicity assessments by means of human health outcomes are rare. Thus risk assessment is particularly difficult to validate for food chemicals, in contrast to medicines, where dosage is likely to be known and a connection between exposure and adverse effects is more likely to be made.

11.2 Sometimes toxic effects are seen in humans, and when the dose is known, these instances can be used to check the accuracy of extrapolations from animal studies. In other cases inadvertent over-exposure generates human toxicity data. Thus, for example, most cases of poisoning by over-dosage with the insect repellent DEET were in young girls, a special sensitivity which was accurately reflected in studies in which it was found to be especially toxic to female rats and to young rats, albeit at very high doses (Verschoyle *et al.*, 1992).

11.3 Two instances of well-defined poisoning incidents in which a known dietary exposure was excessive provide good evidence that the standard assessment was correct. Wu *et al.* (2001) studied four persons who developed acute cholinergic signs after consuming vegetables with abnormally high residues of methamidophos. The residue levels in leftovers from the meals of those poisoned were between 52 and 510 times the JMPR maximum residue limit (MRL) for methamidophos residues in leaf vegetables (MRLs for other vegetables were set at the same or lower concentrations), which suggests that the MRL was not unreasonably high. In a second case, contamination of food with methomyl prior to cooking led to 107 persons being poisoned (Buchholz *et al.*, 2002). In this case the dose consumed by those poisoned could be estimated from the level of contamination of the cooking salt and from salt usage, and the authors were then able to estimate an ED_{50} for production of signs of poisoning (0.09-0.31 mg/kg). This may be compared to the JMPR ARfD for methomyl of 0.02 mg/kg (itself based on a human volunteer NOAEL of 0.1 mg/kg). The clinical poisoning data (necessarily somewhat imperfect) indicate that a sufficient margin of safety was incorporated into the original toxicological assessment. Although five children became ill, adults were more likely to be poisoned than children (odds ratio 3.5) suggesting that children were not an especially vulnerable group in this situation.

11.4 The most difficult challenges for validation of toxicological testing come from delayed or chronic effects, where the cause of the adverse effect may be hard to identify in man. This is particularly true for carcinogens and teratogens, where there is a necessary delay between exposure and detection of effect. The potential for teratogenic effects of the anticonvulsant medicine valproate was first predicted on the

basis of tests carried out in mice, which showed a smaller difference between the minimum teratogenic and minimum toxic doses for valproate than for phenytoin (Brown *et al.*, 1980). This prediction was unfortunately confirmed by subsequent epidemiological studies, which showed increased prevalence of spina bifida and other birth defects in children born to mothers who took valproate during pregnancy (Kalter, 2004).

11.5 In the absence of measures of effect at low exposures it is occasionally possible to use tissue residues (internal dose) to compare animal and human exposures. Thus, concentrations of polychlorinated biphenyls (PCBs) have been measured in brains and adipose tissue of rats used for toxicology assessments (Lilienthal *et al.*, 2000) and also in human still births, the only source of human brain material available (Lanting *et al.*, 1998). Comparing means with means and assuming that the four marker congeners make up about 60% of total PCBs in man, rats at the lowest adverse dose level have about 20 times higher tissue levels than in human stillbirths. Thus an exposure margin of about 20 is obtained for a comparison between mean rat data and a human population at the most susceptible stage of development. However, the relevance of this observation is limited since it cannot be related to functional consequences.

Evaluation of the Default Uncertainty Factor

11.6 Good quality exposure data in humans are available only rarely so that a NOAEL can only exceptionally be estimated from human data. The 10-fold interspecies factor which is applied to the dose expressed on a body weight basis (e.g. mg/kg body weight) has to allow for both toxicokinetic differences in organ blood flow and metabolic activity. This factor has been criticised on a number of grounds. The same generic 10-fold inter-species factor is applied to data from all test species, despite the fact that there are smaller differences in many aspects of physiology and metabolism between dogs or primates and humans, than between rats or mice and humans. It has been suggested that uncertainty factors of about 4 and 10 would be necessary for rats and mice just to allow for differences in renal and hepatic blood flows compared to humans (Renwick 1993). It has been proposed that interspecies comparisons should be on the basis of surface area rather than body weight (Davidson *et al.*, 1986; Calabrese *et al.*, 1992; Kalberlah and Schneider, 1998), which would result in a different default adjustment factor for each species. The appropriateness of the 10-fold inter-species factor is difficult to assess without dose-response data for toxic responses in humans, and fortunately such data are rare. Animal data would not be used for risk assessment in cases where relevant data on humans are available.

11.7 The use of a 10-fold uncertainty factor for interspecies differences is supported by the data on paracetamol, which undergoes metabolic bioactivation via CYP2E1 and CYP1A2. Differences in this activation step account, almost entirely, for the ten-fold higher sensitivity to paracetamol of mice, compared to rats (Tee *et al.*, 1987). A single dose of 1g has been taken safely by millions of people for its analgesic properties. There are marked species differences in sensitivity to the hepatotoxicity of paracetamol due to differences in the rate of conversion to its cytotoxic metabolite. The mean activity of humans is less than that of the rat and even the most sensitive humans have lower metabolic activity than the mouse at activating paracetamol (Boobis *et al.*, 1992). The NOAEL in rat is >1000 mg/kg, whilst in mice it is 150 mg/kg. It has been estimated that the NOAEL in humans is around 200 mg/kg, although there is considerable inter-individual variability that relates to differences in the rate of activation, the amount of glutathione available for conjugating the cytotoxic metabolite and the rate of its clearance. There are

some subjects who can tolerate almost 1000 mg/kg without harm (Prescott, 2001). It is likely that the NOAEL in the average human is greater than 200 mg/kg, as this estimate is from patients who were hospitalised (Boobis *et al.*, 1992). These data suggest that humans are of similar sensitivity to the more sensitive species and support the use of a 10-fold uncertainty factor, which assumes that humans are 10-times more sensitive than the most sensitive test animal species.

11.8 There have been numerous reviews of the appropriateness of the 10-fold factor for human variability, based on human variability in kinetics and dynamics (Hattis *et al.*, 1987; Hattis and Minkowitz, 1996; Naumann *et al.*, 1995; Naumann *et al.*, 1997; Renwick and Lazarus, 1998; Silverman *et al.*, 1999; Suh *et al.*, 1999; Hattis *et al.*, 1999; Veirmeire *et al.*, 1999; Dorne *et al.*, 2001a,b; 2002; 2003a,b; 2004a,b, 2005; Naumann *et al.*, 2001). Attempts have been made to quantify what proportion of the human population would be protected by these safety factors. The analyses of Renwick and Lazarus (1998) indicated that the 10-fold uncertainty factor for human variability would cover the vast majority of exposed individuals assuming a normal or a log-normal distribution[3] (such models would not cover 100% of a population unless the uncertainty factor is infinity). Gaylor and Kodell (2000) suggested that these factors should ensure that the resultant health based guidance value (e.g. ADI) would be below the threshold value for harm in between 92 and › 98% of the human population. The studies of Dorne *et al.*, (2003 a,b; 2004 a,b; 2005) used a log-normal distribution to model human variability in kinetics and concluded that for compounds dependent upon simple monomorphic processes for their kinetics, an even higher percentile of the population would be protected by a toxicokinetic uncertainty factor of $10^{0.5}$ (see below). The data on therapeutic drugs indicate that the current uncertainty factor is a reasonable default value and would cover the normal human population to greater than 99%. However the usual 10-fold factor would not allow adequately for human variability when there is a genetic polymorphism in the main (›70% of the dose) route of elimination. Also differences between healthy adults and some subgroups of the population (such as preterm infants), may not be covered adequately. Differences between human neonates and human adults would not need to be allowed for by the uncertainty factor for human variability if there were a developmental study in neonatal animals, because the interspecies comparison would take into account any risk related to immaturity, providing that the neonatal rat was at least as immature as the neonatal human.

Methylmercury

11.9 Methylmercury is an example of a compound where there are toxicological data in both humans and animals (COT, 2003). Knowledge of the neurotoxicity of methylmercury in human adults predated any systematic study of methylmercury toxicity in animals. The first animal studies (in the 1940s) reproduced the neurotoxicity seen in adults, but potential developmental neurotoxicity was not investigated. Several outbreaks of unexpected human developmental neurotoxicity were described subsequently. Developmental neurotoxicity was reproduced in several species of experimental animal, although the database in animals is, by comparison with regulated chemicals, defective in particular with respect to developmental neurotoxicity. Nevertheless, had it been necessary to set a Provisional Tolerable Weekly

3 The distribution of many biological variables are described by such models. An example is the height of humans, for which a normal distribution give a reasonable discription of the distribution of heights around the average but is less reliable for describing extremes of height. A normal distribution suggests that a few people would be extremely tall (eg taller than 30 feet), which is clearly not the case.

Intake (PTWI) on the basis of the animal data, it is likely that it would have been similar to or below that established by JECFA on the basis of the human data. This analysis provides support for the default 100-fold uncertainty factor, as described in detail in Appendix 3.

12 Other Approaches

12.1 In the conventional "threshold" approach that is frequently used in toxicological assessments, it is assumed that none of the adverse effects of the substance being assessed occur below a threshold dose and NOAELs from studies are used to identify health-based guidance values/reference doses. There are several alternative approaches that can be used in certain circumstances, such as situations in which a threshold cannot be assumed (eg. genotoxic carcinogens) or when there are insufficient data available to identify NOAELs for all of the critical toxicological endpoints. Some of these alternative approaches are discussed and compared with the conventional threshold approach in the text below and at the end of this Chapter in Table 12.1.

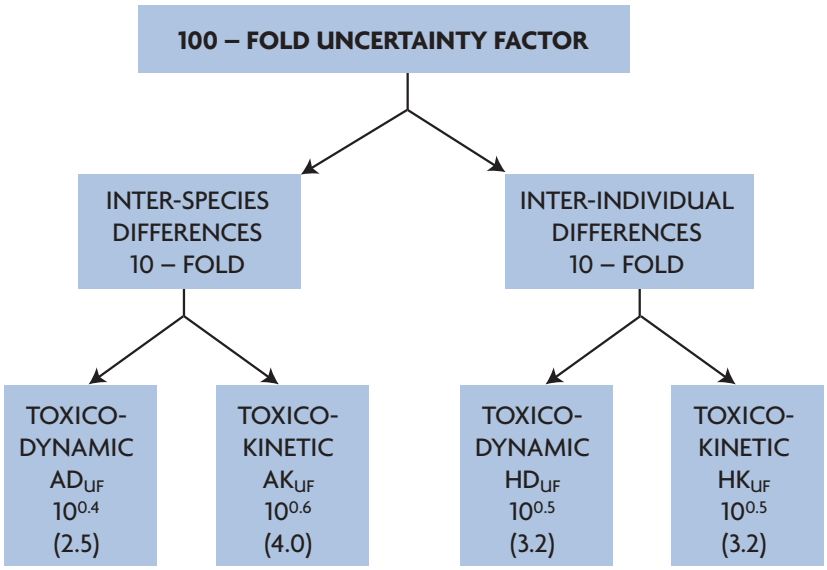
Refining the Uncertainty Factor Approach

Chemical Specific Adjustment Factors (CSAFs)

12.2 The recognition that the 100-fold safety/uncertainty factor could be considered to represent two 10-fold factors allowed some flexibility in its application, because different factors could be applied to the NOAEL from a study in humans and from a study in animals. The concept of chemical-specific adjustment factors (CSAFs) was introduced to allow appropriate data on species differences or human variability in either toxicokinetics or toxicodynamics to modify the relevant 10-fold uncertainty factor.

12.3 Incorporation of toxicokinetic or toxicodynamic data becomes possible if each 10-fold factor is sub-divided into appropriately weighted factors (Figure 12.1).

Figure 12.1. Subdivision of the 10-fold uncertainty factors to allow for species differences and human variability in toxicokinetics or toxicodynamics (based on IPCS, 1994)



Chemical specific data can be used to replace a default uncertainty factor (UF) by an adjustment factor (AF). A – animal to human; H – human variability; D – toxicodynamics; K – toxicokinetics

12.4 The weighting given in the subdivision of each 10-fold factor was discussed at an IPCS meeting in 1994, and the values shown in Figure 12.1 were proposed as suitable defaults. The inter-species subdivision was unequal to reflect the inherent physiological differences between rodents and humans in basic processes, such as renal and hepatic blood-flow which have been discussed above. The even subdivision of the human variability factor was based on an analysis of data on therapeutic drugs. The total uncertainty factor that would be used in risk assessment would be the composite value obtained on multiplying the CSAF, used to replace one of the four sub-factors, by the remaining unmodified values for which suitable data were not available. In this way chemical specific data in one area, such as an inter-species PBPK model, could be introduced quantitatively into the derivation of the ADI/TDI, and data would replace uncertainty.

12.5 The types of chemical-specific data that would be suitable to replace one of the default values has been discussed at a series of international meetings, held as part of an IPCS project on the Harmonization of Approaches to the Risk Assessment of Risk from Exposure to Chemicals. The proposals have been published and made available for comment on the IPCS website (IPCS, 2005).

12.6 The interspecies default factors could be replaced by data that adequately defined the difference in the mean parameter estimates between the test species in the study giving the NOAEL and adult humans. The choice of the appropriate parameter estimate for toxicokinetics or toxicodynamics would relate to the nature of exposure in the study giving the NOAEL and the critical effect that was the basis for defining the NOAEL. Adequate data would provide a reliable measurement of the central tendency for the parameter in the test species at the NOAEL and in humans at relevant intakes, i.e. the predicted intake (see IPCS, 2005 for further details).

12.7 The default factors for human variability could be replaced by data that defined the variability in the relevant parameter estimates in healthy human adults, including the influence of any functional genetic polymorphism, and in addition the variations between different age groups if necessary (see above). Also allowance could be made for the fact that for highly lipid soluble compounds the ratio of tissue concentration to body burden is likely to be influenced by fat as a proportion of body weight. A mathematical model of human variability would need to be defined and a percentile of the distribution, such as a 90th, 95th or 97.5th percentile selected to replace the default (see IPCS, 2005, for further details).

12.8 The concept of subdividing the 10-fold uncertainty factors has been used in two recent evaluations. In the case of dioxins, the interspecies comparison was based on body burden, such that the default interspecies kinetic uncertainty factor was not needed and AKUF became 1. It was considered that the tissue response in the most sensitive human would be no greater than the test species, so that ADUF and HDUF became 1. In consequence the total composite factor became 1 x 1 x 1 x 3.2 = 3.2. In this case the extensive database on dioxins could be used to provide a numerical interpretation in 3 of the 4 areas of uncertainty (JECFA, 2002). In the case of methylmercury, the critical data were derived from epidemiological studies so that interspecies factors were not relevant (see Chapter 10). The default factor for human variability in toxicodynamics was considered not to be necessary because the NOAEL/BMD was based on concentrations in blood or hair detected in a large epidemiology database of the most sensitive subgroup of the population, so that HD_{UF} became 1 (IPCS, 2005).

12.9 The proposed toxicokinetic/toxicodynamic approach has not been designed to accommodate information from *in vitro* and *in silico* studies. Further consideration of how such data may be taken into account is needed.

Benchmark dose (BMD)

12.10 The benchmark dose (BMD) is defined as the dose producing a predetermined (e.g. 5% or 10%) incidence of an adverse effect, and is derived by statistical modelling of the dose response data (EPA, 2003). Both quantal data (e.g. lethality, presence of tumours) and continuous data (e.g. change in liver weight in a dose group) can be evaluated with the BMD approach. The lower 95% confidence interval of the BMD is referred to as the BMDL. Early references to this approach used the term BMD for the lower confidence limit, but BMDL is now preferred as it is considered clearer (Crump, 1995; EPA, 2003) and this is being adopted more widely (FAO/WHO, 2005; EFSA, 2005a). EFSA has set up a Scientific committee on the Use of the Benchmark Dose Approach in Risk Assessment to advise on the advantages and limitations of the use of the BMDL in place of the NOAEL and to provide an opinion on the preferred approach to be used for risk assessments conducted by EFSA's Panels and Expert Groups (EFSA, 2005b).

12.11 The BMD approach is used as an alternative to the NOAEL. It makes use of all of the dose-response data (Crump, 1984). The NOAEL is an observed value that is dependent on study design and which makes little use of the effects detected at doses above the NOAEL, other than to identify the nature of the hazard. The BMD approach avoids the problems associated with the NOAEL (see Chapter 7) by fitting a mathematical model to all of the dose-response data to derive the dose associated with a predefined level of response (the benchmark response [BMR]) and its associated uncertainty. In consequence the BMD is less dependent on study design, dose spacing or even group size, while well conducted studies with low variability are "rewarded" with higher BMDL values. Another major advantage of the BMD approach is that an estimate of the BMD can be obtained using data from an otherwise satisfactory study in which a NOAEL was not found and all dose levels produced some level of response above controls.

12.12 The validity of the BMD approach is dependent on the number of points to which the model was fitted, and ideally there should be 3-4 doses showing graded responses. One problem with the BMD is that current study designs and testing guidelines are designed to provide reliable estimates of the NOAEL rather than defining the shape of the dose-response relationship. The BMD approach offers no advantages in cases where the response is observed at the top dose only. An additional problem is selection of the level at which the BMR should be set. In the typical subchronic and long-term study on a pesticide, the highest dose used is a maximum tolerated dose (MTD) and the lowest dose is designed to be a no-effect dose, so that the dose response curve is based upon the high and medium doses only. This situation is not ideal for the calculation of a BMDL.

12.13 The BMD approach is reliant on statistics and the accuracy and validity of the model being used. As such, it is important to select the appropriate model structure for the data being analysed. For threshold effects, the BMDL can be used as an alternative to the NOAEL as the basis for calculating an ADI or TDI, by the application of the usual uncertainty factors (see above but the use of a BMDL raises a communication difficulty, because it is not a dose apparently without any effect, but the lower confidence interval on the dose associated with a predefined response above controls). In reality, the

outcome would be very similar to that derived using a NOAEL, but with a better characterised starting point. Indeed, the NOAEL may not be a level of intake at which there is a complete absence of any response, it is simply the level at which a response is no longer observable under the experimental conditions (see also Chapter 7). For non-threshold effects, such as (it is assumed) carcinogenicity produced by a mutagenic compound, the BMDL can be used as the starting point for linear low-dose extrapolation or for calculation of a margin of exposure (FAO/WHO 2005).

Characterising Uncertainty

Margin of Exposure approach (MOE)

12.14 In some instances, where there are too many gaps in the toxicological data to allow a Tolerable Daily Intake to be established, scientific advisory committees have used a MOE approach, for example in commenting on dietary exposure to certain brominated flame retardants (COT, 2004b). The margin of exposure (MOE) is calculated as the ratio between a defined point on the dose-response curve for the adverse effect, often the NOAEL or BMD, and the human intake.

12.15 In evaluating whether an MOE is high enough to conclude that a chemical is unlikely to cause harm the usual toxicokinetic and toxicodynamic uncertainties related to species differences and potential human variability associated with the data must be taken into account. Choosing the acceptable margin between the critical point on the dose response curve and exposure presents similar difficulties to choosing an UF in an ADI calculation. When the starting point for the derivation of an MOE is the NOAEL or the BMD, there is very little difference in risk assessment using the MOE as compared with the ADI, and the choice is often as much historical as scientific. It should be noted that with the MOE, the uncertainty factor is explicit (the MOE) whilst for the ADI it is integral in the calculation since it is the factor used to derive the ADI from the critical NOAEL or BMD. Nevertheless with both approaches the uncertainty factor should be made explicit by toxicologists and risk managers.

12.16 In calculating the MOE, there is no necessity to use the NOAEL, and any appropriate defined point on the dose-response curve would be suitable. The MOE can be used without making any implicit assumptions about safety. This approach can be used for contaminants where there is evidence of a threshold response but not enough evidence to derive a TDI (in which case an MOE larger than 100 would normally be required), or for non-threshold effects where the response at human exposures is not known, and cannot be modelled appropriately. For non-threshold effects, the MOE provides a simple and practical approach that avoids the scientific uncertainties associated with the selection of a mathematical model for low-dose extrapolation, and doubts about its biological relevance at low doses. The reliability of the MOE set for a substance would depend upon the adequacy of the database, and the possibility of cytoprotection and repair processes might need to be considered. When used for non-threshold effects, the MOE is intended to provide risk managers with more helpful information than the ALARP approach described in Chapter 3. It can be useful as a tool for ranking the possible risks from a series of hazards or exposure situations.

12.17 The MOE approach can be used in the risk assessment of mixtures of chemicals with similar modes of action but different potencies, by calculating the total margin of exposure (MOE_T). Here the combined

MOE (MOE_T) is the reciprocal of the sum of reciprocals of the MOEs for the various compounds (Wilkinson *et al.*, 2000).

12.18 It is often said that the margin of exposure that would be considered acceptable is a societal judgement and should not be determined by risk assessors alone. Indeed, use of the MOE can enable complete separation of risk assessment from risk management. The risk assessor would provide the risk manager with the margin of exposure and the risk manager would decide on its acceptability. However, risk assessors have the responsibility to inform risk managers on the nature of the hazard, at the critical and possibly other endpoints, the quality of the data, the uncertainties inherent in the data used for intake estimates and the magnitudes of the margins of exposure. In evaluating the potential health consequences of the MOE for a given exposure, due consideration needs to be given to the toxicokinetic and toxicodynamic uncertainties in extrapolating across species and individuals.

Probabilistic modelling

12.19 Probabilistic methods use mathematical or statistical models to express the relationship between factors and variables relevant for exposure assessment, hazard characterisation or risk assessment. Variability and uncertainty in the input values of the variables is expressed by using probability distributions of values, as distinct from a single (eg. "worst case") value for each variable. Outputs are also expressed in terms of probability distributions that reflect the combined probabilities of the input data (eg. exposure data); it is possible to use this output distribution to estimate the proportion of the population at a particular exposure level. This method may give a more realistic representation of the risk being assessed than current methods which use a single value or "point estimate". In addition, probabilistic modelling facilitates sensitivity analyses of which factors have the most influence on the result. Probabilistic modelling has been used for exposure estimations, where there is some evidence that they provide a more accurate estimate of exposure than the traditional deterministic paradigm, at least with pesticide residues (e.g. Boon *et al.*, 2003). Probabilistic modelling has been little used in toxicological risk assessment in the UK, although these approaches (Elder *et al.*, 2002) are increasingly being used in other countries such as the USA.

12.20 Probabilistic models may be used under a classical or Bayesian statistical paradigm. In the classical model, probability is regarded as a frequency that an event will occur, based on repeated sampling.

12.21 Monte Carlo simulation is the method most commonly used for both classical and Bayesian probabilistic modelling. This involves taking repeated samples from the distribution of the input variables, analogous to doing repeated experiments. Use of a probabilistic model means explicit acceptance of a finite degree of adverse effect, which is not necessarily the case with the NOAEL approach.

12.22 Implicit in probability modelling is the need to define the probability density function of the parameters comprising the model.

12.23 Bayesian statistical methods are based on Bayes' theorem. According to the Bayesian approach, a probability is regarded as how likely it is thought to be, based on judgement as well as observational data, that an event will occur. Hence, unlike other methods, Bayesian methods do not assume that we are trying to identify the "true" value of a parameter, rather the intention is to refine the level of uncertainty about the parameter.

Bayesian theory starts with a prior distribution, ie. a probability density function. This represents the knowledge and assumptions about the event of concern. This prior distribution is combined with the distribution of a new set on data that are obtained, to give a posterior distribution of the updated knowledge. Bayesian methods allow combination of multiple items of information and incorporation of expert judgement. Sensitivity analyses of both the data and the models can be carried out.

12.24 Uncertainties in probabilistic modelling may arise from the choice of range that has been modelled and the confidence in that range. The evaluation of the reliability of the models to extrapolate between animals and humans and to determine the validity of the model is required. In addition, probabilistic modelling is data intensive and for general applicability of the output for exposure estimates random, not targeted, surveillance data are required (COT, 2002b). The outputs are determined by the mathematical model used and it is important for the toxicologist to understand the nature of the model and its constraints. The relationship of the model to the underlying biological response can be unclear. Bayesian methods have the advantage of being able to express variability and uncertainty in numerical form, incorporate lack of knowledge as well as knowledge, and can be easily updated when new data become available. They also offer the potential for enhanced transparency of the decision making process.

12.25 Experience in the use of probabilistic modelling is currently largely confined to exposure assessment. There have been attempts to apply probabilistic modelling to toxicological data such as toxic equivalency factors for dioxins and dioxin-like PCBs in order to reflect uncertainty. Probabilistic modelling of the dose response could indicate the proportions of individuals at risk at a given level of exposure. This possibility has been considered theoretically but has not yet been widely used in regulatory risk assessment (IEH, 1998). However, it has been suggested that probabilistic modelling approaches should be explored and their usefulness evaluated in a parallel exercise comparing these approaches with current methods for selected risk assessment issues (IEH, 1998)

Categorical regression

12.26 This is a mathematical model which uses meta-analysis techniques and which allows incorporation of information from different studies on how the incidence of response increases with dose and how the severity of response increases with dose. Categorical regression uses regression of ordered categories of toxic severity, to estimate the likelihood of a given category of severity at a given dose level. Categorical regression involves statistical regression on the experimental doses associated with various severity categories of overall toxicity (Hertzberg & Miller, 1985; Edler *et al.*, 2002). Categories of severity may be assigned to qualitatively different levels of effect, and the doses and their associated effects from different studies may be combined to predict effect severity. In this way, all categorised adverse effects may be taken into account rather than focusing on the critical effect only. A problem with this approach is that it is often used to combine different effects in different studies and even different species. Although the method provides a global picture, in the end the risk assessment will usually focus on the effect seen at the lowest doses. The greatest use of categorical regression analysis is likely to be when humans are exposed to potentially toxic levels, and information is needed on what effects may be anticipated.

Whole Body Physiologically-Based Pharmacokinetic Modelling (PBPK)

12.27 Use of physiologically-based pharmacokinetic or toxicokinetic (PBPK/PBTK) modelling has the potential to allow the prediction of tissue concentrations of chemicals for tissue dosimetry and to allow extrapolation between routes of administration, species, high to low doses and different exposure scenarios. PBPK/PBTK models are valuable tools for extrapolating findings in animals and other test systems to humans and are accepted as part of hazard characterisation. The COT has concluded that they can be used as part of the risk assessment process, and can be valuable in identifying those parameters exerting most influence on the behaviour of the compound (sensitivity analysis) and as a means of exploring inter-individual variability (COT, 2003a). A simple empirical method of dealing with the problem of extrapolation from data obtained in species of low body mass to higher body mass is to use allometric scaling. Typically body mass to the power 0.75 has been used. Allometric scaling works best for compounds where the elimination is dependent on basal metabolic rate. However many other compounds of concern to COT rely upon specific enzymes or transporters for their toxicity or elimination that do not scale allometrically. Although allometric scaling sometimes reduces inter-species variability, this is not always the case (see Appendix 3, paragraph A3.24), and it is always preferable to use a data-based pharmacokinetic or mechanism-based factor for extrapolation

12.28 The use of PBPK modelling to improve study design is a significant feature of the new system for assessment of agricultural chemicals that has been proposed in the "Agricultural Chemical Safety Assessment" project of the International Life Sciences Institute (Carmicheal *et al.*, 2006; Barton *et al.*, 2006; Doe *et al.*, 2006, Cooper *et al.*, 2006). While PBPK models require considerably more human and animal data than when using empirical approaches, they provide a mechanistic approach to both understanding the temporal behaviour of compounds within the body and predicting what is likely to happen in plasma and tissues over a wide range of conditions.

12.29 A PBPK/PBTK model comprises three components 1) a body of independent physiological, anatomical, and biochemical data – *the system*; 2) *substance-specific data* overlaid on to the system; and 3) the *model structure*, this being the tissues and organs included in the model, their structural complexity and their anatomical arrangement. As knowledge of the system and how compounds interact with it increases, so will the ability to predict the likely behaviour of compounds from relatively limited data on the compound. To provide meaningful predictions, however, it is important to incorporate biological variability and methodological uncertainty in parameter values throughout the modelling process. Unlike with empirical models, compound-specific data from various sources, *in silico*, *in vitro*, and *in vivo*, can readily be incorporated into PBPK/PBTK models. However, it is critical to verify at every opportunity the quality and utility of the input data against events of interest *in vivo*. The PBPK/PBTK model approach is flexible in the sense that it has the potential to be continuously updated in the light of new information, whether physiologic, disease, or compound related.

12.30 PBPK/PBTK models aid in more precise and informative prediction of biodisposition in humans from *in vitro* data, and in the likely behaviour in different subpopulations. PBPK/PBTK models also allow the possibility of examining plausible and likely outcomes in situations where there are severe ethical constraints to experimentation, eg pregnancy. However, there is as yet no consensus as to the means or extent of validation required before a PBPK/PBTK model can be used for regulatory purposes.

12.31 The power and utility of physiologically based modelling would be further increased when linked with mechanistically based pharmacodynamic and toxicodynamic models as well as probabilistic exposure models. When the PBTK model is linked to measurements of target organ response, such as enzyme induction with TCDD, the resulting model covers both kinetics and dynamics and is a PBTK/TD (kinetic/dynamic) model.

12.32 Obstacles to the wider use of PBPK/PBTK modelling include lack of user-friendly modelling software, lack of appropriate and easily accessible relevant physiological and related databases, and, of importance, lack of adequately trained researchers in such modelling. All, however, are soluble if there is willingness to address these obstacles (See Appendix 4). The Computational Toxicology Section (CTS) of the Health and Safety Laboratory, Buxton, has initiated a project to develop practical and rapid PBPK models, facilitate dialogue with industry and regulatory bodies and contrbute to the development of Good Modelling Practice.

Use of Systematic Review and Meta-analysis Methods

12.33 As discussed in Chapter 8, although systematic review and meta-analysis techniques have been widely employed in evidence-based practice and clinical trial research and increasingly in epidemiology, their use in toxicology is still limited. A recent exploration has been the development of methods for combining research from studies of different designs (cross-design synthesis) including approaches for combining epidemiological and toxicological data.

12.34 Most risk assessments for setting health-based guidance values are based on narrative reviews of toxicological literature. An essential difference between narrative and systematic reviews is the requirement for the latter to set out in a standardised and explicit way the target research questions, methods and results, including any assumptions and judgements made. This provides transparency of methodology, allows reproducibility and facilitates updating. These methods should improve the elucidation and evaluation of sources of uncertainty and variability and ensure that decisions about these are recorded in a systematic way. Several recent articles in the Lancet and British Medical Journal have advocated the use of systematic review and meta-analyses for assessing the literature on animal experiments (Sandercock and Roberts, 2002; Roberts *et al.* 2002; Pound *et al.* 2004; Khan and Mignini, 2005; Macleod *et al.*, 2005).

12.35 Much of the focus of systematic reviews and meta-analyses to evaluate animal experiments has been on their use to decide whether to start clinical trials of an intervention in humans. However, in the last five years there have been more published meta-analyses, and to some extent systematic reviews, where an environmental chemical is the main focus.

12.36 As part of systematic review it may be advantageous to synthesise (meta-analyse) results from several studies quantitatively. There are a large number of methods available for such quantitative synthesis of evidence including classical frequentist and Bayesian methods (reviewed in detail by Sutton *et al.*, 2000). Meta-analyses offer the potential advantages of greater power, more precise estimates, a framework for investigation of sources of heterogeneity between studies and, using Bayesian methods, for expert

judgement and/or evidence from sources of evidence other than the study being analysed to be incorporated (Speigelhalter *et al.*, 2000).

12.37 Meta-analysis of animal experiments would facilitate assessment of heterogeneity and publication bias and would allow combination of data from experiments using different animal species and strains and exploration of the sensitivity of results to differences in these variables. This advantage has not so far been commonly reported in the published meta-analyses of animal experiments. There is scope for future work in this area. In the epidemiological area meta-analyses facilitate investigation of the influence of confounding and effect modifying variables on the meta-analysed estimate and exploration of the sources of bias outlined in Chapter 8. There are also well-developed methods for exploring the issue of publication bias which is rarely addressed in reviews of toxicological literature.

12.38 The methylmercury example described earlier and in Appendix 3 is one where health-based guidance values have been set using human data but there is also a considerable amount of animal data. There would be an opportunity to apply systematic review and meta-analysis techniques to these data, for example, to synthesise the animal data, the human data and potentially the animal with the human data. Elicitation and incorporation of expert judgement and differing prior beliefs from committee members, for example on their choice of key/important animal studies, preference for data from certain animal species or strains, judgement on quality of animal and/or human studies could be explored using Bayesian methods. These approaches address the question 'what if?' e.g. what would the outcome of the risk assessment be if the committee chose a different key study, made certain assumptions about a source of uncertainty etc. They also introduce transparency and reproducibility by setting out explicitly the assumptions and the decisions made and by exploring the impact of sources of uncertainty and variability.

Table 12.1: Strengths. Limitations and weaknesses of several alternative approaches (based on Edler *et al.*, 2002)

Approach	Strengths	Limitations and Weaknesses
Structure-Activity Relationships (SAR) and Threshold of Toxicological Concern (TCC)	Avoids unnecessary animal testing	Assumes that structure predicts toxicity. Depends on current exposure estimates for the population.
Threshold	Is simple to apply and readily understood	Assumes the existence of a threshold. The NOAEL does not exclude biologically significant effects below the sensitivity of the tests. The value of the NOAEL depends on the experimental conditions such as group size, sensitivity of measurement of the adverse effect, and dose spacing. Does not make full use of the dose-response information. Uses default uncertainty factors.

Approach	Strengths	Limitations and Weaknesses
Chemical-Specific Adjustment Factor (CSAF) modelling	Chemical-specific data can be incorporated to reduce uncertainty.	Depends on the validity of the subdivision of the 10-fold factors. Is a data intensive method.
Non-threshold	Linear extrapolation is simple to apply.	Linear extrapolation is thought to be highly conservative. The Linearised Multi-Stage (LMS) model cannot be validated as a model for low doses and extrapolation is model-dependent. Differing balances between reactivity and repair between low and high doses are not accommodated.
Benchmark Dose Level (BMDL)	Makes full use of the dose-response data. Allows confidence limits for point estimates. An optimal experimental design may allow reduction of the number of animals tested (does not require a large number of animals per group).	Obtaining consensus defining a benchmark response level for the adverse effect (eg. 5 or 10%) is difficult. Is not applicable to studies with few dose groups or not showing a range of responses.
Probablistic risk assessment	Uncertainties associated with all aspects of the quantitative methods of the risk assessment process can be taken into account. Appropriate chemical-specific information can be incorporated to reduce uncertainty. Provides effect estimates at actual exposure levels.	Requires use of default distributions in most cases.
Categorical regression	Takes all studies into account and not only the most sensitive ones. Allows the prediction of a severity effect category at a particular dose (eg. above ADI).	Requires toxicological judgement for the categorisation. The interpretation of fitted model (different endpoints, observer variation, etc.) is difficult.

Approach	Strengths	Limitations and Weaknesses
Physiologically-Based Toxicokinetic (PBTK) modelling	Is able to model the time course of the amount of the active compound at the target site. Is possible for any species, dose and for different exposure (eg. route to route extrapolations) and lifetime conditions. Allows extrapolation from animal to human without having to have human data. Allows target organ dose-response relationships to be used for low dose extrapolation.	Is a data intensive method. Does not address the toxicodynamics.

13 Conclusions and Recommendations

Conclusions

13.1 The conclusions are set out below, under the respective terms of reference:

To review the evidence of the bases and range of variability in response to toxic chemicals

13.2 Variability in response to chemicals is determined by the fate of the chemical within the body (toxicokinetics) and the toxicity of the chemical and its metabolites (toxicodynamics). Variability in both toxicokinetics and toxicodynamics arises from a combination of factors that are inherent to the individual (or organism), and other factors that relate to the physiology and environment of the individual and which change over time. Inherent characteristics include species, sex and genotype. The modulating factors include age, stage of development and functional maturation of organs and systems, co-exposure to other agents and compounds (e.g. nutrients), lifestyle, and environmental factors, and disease. In principle, variability is measurable, and any lack of knowledge of variability is a source of uncertainty.

13.3 Recent scientific advances have led to greater insight into genetic factors responsible for susceptibility. It is now becoming possible to explain some variability in terms of genetic polymorphisms and post-genomic molecular biology. However, genetic heterogeneity and gene expression are not new forms of variation. They have always existed as part of the underlying differences between individuals, and as such would have been part of the variability that has informed the development of current methods that are applied to deal with variability in risk assessment.

13.4 The range of human variability in response to chemicals cannot be measured directly in all sectors of the population. It is inferred from studies in different animal species, and from knowledge of the differences between humans and animal models in toxicokinetics and toxicodynamics.

13.5 Each source of variability results in a distribution of activity or functionality amongst individuals of a population, leading to either increased or decreased susceptibility to the toxic effect. The overall response to a toxic chemical is determined by the combination of the many different sources of variability. The factors contributing to variability are, unless linked, unlikely to all act in the same direction.

To consider sources of uncertainty in hazard identification and characterisation

13.6 Uncertainty in hazard identification or characterisation relates to incomplete knowledge of the relevance of the results of studies conducted in animals and experimental models, or of human populations, and of possible effects that have not been adequately investigated or recognised.

13.7 The few available direct data relating to human variability are largely derived from studies in young men. Thus the extent to which these data reflect the susceptibilities of women, older people, the conceptus, or children is uncertain.

13.8 Often there is uncertainty about the association of early exposure with particular health effects later in life. Improved understanding of the pathogenesis of such effects might enable identification of early predictive markers of significant adverse effects that would reduce this uncertainty.

13.9 There is more uncertainty in the hazard identification and characterisation of contaminants and natural constituents in foods because, unlike food additives and pesticides, they are not subject to a formal approval process requiring systematic studies to support safety assessment.

13.10 Another source of uncertainty relates to interpretation of studies giving apparently contradictory results with no obvious explanation. There is a need for an agreed robust mechanism for assessing the results of studies that give contradictory results, and demonstrating clearly how the hazard characterisation resolves such problems.

To consider the appropriateness of uncertainty factors customarily used to extrapolate toxicological data from animals to humans

13.11 Differences between the animal species used in laboratory experiments and humans derive from anatomical and physiological differences, as well as the variation in genetic factors that occurs within a species. Data from the available research in which compounds have been studied in both animals and man suggest that the default uncertainty factor of 10 allows adequately for interspecies differences.

13.12 The question of special vulnerability of the developing nervous system to neurotoxicity is addressed by current regulatory testing with specific consideration of neurobehavioural and neurodevelopment outcomes. Similarly, data derived from developmental and reproductive toxicity studies in animals can be extrapolated to humans in considering other effects on the fetus and infant. Results suggest that the current approaches and uncertainty factors are adequate. However, it is recommended that this area be kept under review.

To consider the appropriateness of uncertainty factors customarily used to allow for variation within the human population, including subgroups such as children.

13.13 Inter-individual differences in the activity of xenobiotic metabolising enzymes are often characterised in well defined populations of subjects, focusing only on the pathway of interest. Whilst 10-fold or greater differences have been demonstrated between groups, there are frequently no comparable differences in the overall kinetics of the parent chemical, because of compensation by alternative pathways.

13.14 The default uncertainty factor for interindividual variability has been explored empirically on a number of occasions. This has usually been performed with pharmaceutical agents, but these studies can be related to other chemicals, and they suggest that the default uncertainty factor is generally appropriate.

13.15 With a few exceptions, particularly susceptible subgroups cannot be identified by genotype. Some subgroups are potentially vulnerable due to physiological, dietary or environmental factors. With respect to infants and children, it is recognised that the young can be either more suceptible or less susceptible than adults to the toxicity of particular substances. Since more information on newly introduced human pharmaceutical agents will be expected to be derived directly from observations of treated children, it should in future be possible to test the adequacy of current uncertainty factors in protecting young children.

13.16 The possibly increased susceptibilty of the elderly and the consequences of a lifetime of exposure are representatively investigated in chronic toxicity studies. Even so, there is a need for better characterisation of the uncertainties related to possible altered susceptibility arising from environmental, physiological and metabolic changes during the course of life and in older life. An additional uncertainty factor for this is probably unnecessary in most cases but should be considered and decided during hazard characterisation on a case-by-case basis.

To consider other methods that might be used in setting acceptable or tolerable intakes for chemicals in food, consumer products and the environment

13.17 The COT uses current internationally-accepted methods in its risk assessments. These make good use of state-of-the-art knowledge and of the methodologies available to take account of variability and sub-group vulnerability in toxicological data. Given the wide range in primary data quality and the frequent occurrence of critical data gaps, it is not possible to propose the use of any single approach to risk assessment, but rather it is necessary to continue with the present flexible use of the assessment methodology best suited to the specific data set available. As a continuing process, the COT will consider improving and refining the methods and approaches it uses.

13.18 *In vitro* studies have important roles to play in hazard characterisation and investigations of toxicological mechanisms. However, there remain uncertainties with regard to the extrapolation of the results of *in vitro* studies to humans. Complete replacement of animal tests in toxicology is not possible at present.

13.19 Application of the default 100-fold uncertainty factor, which allows for 10-fold factors each for inter- and intra-species variation continues to be a reasonable approach, in the absence of better information. In some instances, e.g. if there is good evidence that humans are not more sensitive than animals, application of the full 100-fold factor is not necessary. Subdivision of the default uncertainty factors to incorporate chemical-specific toxicokinetic and toxicodynamic adjustment factors should be used, whenever data allow. If chemical-specific adjustment factors are used, the adequacy of the remaining default factors should be explicitly considered.

13.20 Statistical and modelling approaches, including physiologically-based pharmaco- or toxicokinetic models, have been used to refine the risk assessment process. Greater use of such methods, when suitable data are available, would support a more systematic approach to risk assessment. Probabilistic models could be used to explore and quantify uncertainty.

13.21 Description of assumptions and uncertainties in the evaluation is important for transparency of the risk assessment:

- Systematic reviews of the relevant toxicology and epidemiology literature are important tools in hazard identification and hazard characterisation and for the presentation of data.

- There should be a description of the criteria for inclusion or exclusion of studies in a review and details of the uncertainties and variabilities in parameters of interest in both the test subjects (eg. laboratory animals or human cohorts) and the human population of interest (eg. consumers or exposed workers).

- The choice of critical event used to set guidance values should be justified.
- The validity and robustness of biomarkers of exposure, intake, susceptibility and outcomes should be discussed, along with environmental and lifestyle factors that might impinge on these factors.
- Vulnerable groups of people should be identified.

To consider how to express the level of confidence that one can have in the risk assessment

13.22 The degrees of variability and uncertainty at each stage of a particular risk assessment should be clearly described and communicated to those involved in risk management. This should include identification of whether all relevant responses were investigated. Particular attention should be given to stating assumptions and subjective elements in the risk assessment, justification of the choices of uncertainty factors used, and of the selection of the adverse health effects used as the basis for risk assessment. Transparency in these factors aids an informed assessment of uncertainty and enables risk managers to communicate this to stakeholders. Furthermore, such transparency is particularly important in reconciling differences in risk assessments reached by different expert groups.

Recommendations

13.23 There is a need to introduce methods to increase the transparency and reproducibility of hazard identification and characterisation. Several recommendations are made for future areas of research and changes in policy to ensure such transparency and reproducibility.

13.24 Research needs relate to the following areas:

Addressing the best use of existing data:

- Exploration of methods for assessing the quality of the toxicological evidence and the sources of uncertainty and variability.
- Development of a framework for transparent expression of uncertainty in hazard characterisation, such as addressing and identifying critical data gaps.

Vulnerable sub-groups:

- Improved understanding of the relevance to susceptibility of the genetic polymorphisms that have been identified in human populations.
- Evaluating whether there are specific subgroups not protected by the default uncertainty factors, due to genetic, physiological (e.g. early and older life) or environmental sources of variability.
- Developing valid mechanism-based biomarkers of uptake, effect and susceptibility that would help to identify subgroups at risk.

- Better characterisation of hazards to older people to determine whether current uncertainty factors are appropriate.

Mixtures of substances:-

- Improve understanding of the combined effects of chemicals occurring in food.

13.25 In relation to policy and practice, it is recommended that:

Hazard characterisation:

- Hazard identification and characterisation should take into account variability and uncertainty, using a systematic approach that will facilitate transparency and confidence.

- Greater use should be made of statistical and modelling approaches, including probabilistic and physiologically-based pharmaco- and toxicokinetic models. Use of such methods, when suitable data are available, would support a more systematic approach to risk assessment allowing for variability within the human population.

- Subdivision of the default uncertainty factors to incorporate chemical-specific toxicokinetic and toxicodynamic adjusted factors should be used, whenever data allow.

Risk communication:

- The development of a framework for transparent expression of uncertainty in hazard characterisation would enable COT and other committees that perform toxicological evaluations to improve communication of the sources of variability and uncertainty in their risk assessments. Particular attention should be given to describing assumptions and subjective elements of the risk assessment, clearly describing where contradictions in information occur and how the resultant uncertainty is resolved.

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Appendix 1

Tests currently used in regulatory toxicology

A1.1 Table A.1 list those tests currently carried out in routine toxicological evaluation of food chemicals. Some of these tests do not yield NOAELs and cannot therefore be directly used for risk characterisation. Some of these are used for other regulatory purposes, including those for acute toxicity, skin and eye irritancy and skin sensitisation. Other studies, such as tests of absorption, distribution, metabolism and excretion and mutagenicity data will help interpret and evaluate toxicity studies that provide NOAELs.

A1.2 It should be emphasized that not all studies may be required with all regulated chemicals and that a sponsor may provide a reasoned statement explaining why he considers certain tests not to be required. In the case of food contaminants and natural constituents many of the studies will not be available.

A1.3 The vast majority of the studies will have been carried out in rodents, rabbits or guinea pigs. Some study types should be performed in more than one species, with rats being almost always one of the species. Dogs are usually used as a non-rodent species for repeat-dose toxicity studies of pesticides and veterinary medicines, rabbits are usually used as the non-rodent species for developmental studies and mice are often used as a second species in carcinogenicity studies. Primates are rarely used.

Table A1. Current tests and endpoints used in toxicology

Study type	Endpoints measured
Tests of absorption, distribution, metabolism and excretion (ADME)	Absorption and excretion rates, test material concentration in body fluids and organs, nature of metabolites
Acute (single dose)	Clinical signs, mortality
Irritancy	Skin irritancy Eye irritancy
Sensitisation	Skin sensitisation
28-day study	Clinicals signs, mortality Body weight, body weight gain Food consumption Water consumption Hematology Clinical chemistry Urinalysis Organ weights Macroscopic/microscopic histopathology
90-day study	Clinical signs, mortality Body weight, body weight gain Food consumption Water consumption Hematology Clinical chemistry Urinalysis Organ weights Macroscopic/microscopic histopathology

Study type	Endpoints measured	
2 year/lifetime study	Clinical signs, mortality Body weight, weight gain Food consumption Water consumption Hematology Clinical chemistry Urinalysis Organ weights Macroscopic/microscopic histopathology	
Developmental toxicology	Dams	Clinical signs, mortality Body weight/weight gain Food consumption Water consumption Macroscopic/microscopic
	Histopathology	Fetal data Numbers of corpora lutea, Implantations Numbers of viable fetuses and Resorptions Sex ratio Fetal and litter weights Skeletal and visceral examination
Reproductive toxicology (multigeneration study)	Parental	Clinical signs, mortality Bodyweight, body weight gain Fertility Sperm analysis Macroscopic pathology Histopathology of reproductive organs
	Litter/pup data	Litter size Numbers of live/dead pups Pup sex Pup weight Pup organ weights Pup macroscopic/microscopic pathology Growth and development post-weaning, including sexual maturation
Mutagenicity studies	Studies *in vitro* Studies *in vivo*	

Appendix 2

Polymorphic xenobiotic metabolising enzymes and their role in susceptibility to disease and toxicity

Gene	Wild type	Common variants	Consequence of polymorphism	Comments	Recent review(s)
CYP1A1	CYP1A1*1	*Msp*I RFLP in 5′ flanking region	Increased inducibility	Functional effects unproven; no clinical effects demonstrated. Majority of variability is associated with regulatory factors.	(Wedlund 2000; Ingelman-Sundberg 2001; Lin and Lu 2001; Meyer and Gut 2002; Burk and Wojnowski 2004; Ingelman-Sundberg 2004; Keshava *et al.*, 2004; Munafo *et al.*, 2004; Zanger *et al.*, 2004; Malaiyandi *et al.*, 2005; Rettie and Jones 2005)
		Point mutation at codon 462	Altered haem-binding region leading to 7-fold increase in activity		
CYP1A2	CYP1A2*1A	CYP1A2*1F	Increased inducibility	Slight increase in risk of myocardial infarction (Cornelis *et al.*, 2004). Lack of response to the antipsychotic clozapine in a small study (Eap *et al.*, 2004).	
CYP1B1	CYP1B1*1	CYP1B1*2 CYP1B1*3 CYP1B1*4 CYP1B1*5 CYP1B1*6 CYP1B1*7	Difficult to interpret due to complex population distribution of haplotypes	Candidate gene for congenital glaucoma. Not shown to be associated with susceptibility to cancer induced by oestradiol exposure, smoking or PAHs.	
CYP2A6	CYP2A6*1	CYP2A6*2 CYP2A6*5 CYP2A6*7 CYP2A6*8 CYP2A6*10	Defective enzyme	Affects reaction to nicotine: differential effects on smoking initiation, conversion to dependence, amount smoked during dependence and cessation. Most marked effect on smoking cessation.	
		CYP2A6*4	Gene deletion		
CYP2B6	CYP2B6*1A	CYP2B6*1H CYP2B6*6B	Reduced activity	In association with alcohol consumption, affects clearance of bupropion.	
CYP2C8	CYP2C8*1	CYP2C8*3	Reduced activity	Homozygotes or double heterozygotes for CYP2C8*3 and CYP2C9*3 (see below) alleles have extremely low ibuprofen clearance rates (Garcia-Martin *et al.*, 2004).	

Gene	Wild type	Common variants	Consequence of polymorphism	Comments	Recent review(s)
CYP2C9	CYP2C9*1	CYP2C9*2	Reduced affinity for cytochrome P450 reductase	Numerous alleles generate >20 possible haplotypes but CYP2C9*2 and CYP2C9*3 make greatest contribution to variable warfarin requirement. There is an increased risk of bleeding during stabilisation phase of warfarin treatment in CYP2C9*3/*3 homozygotes. Studies with recombinants indicate reduced intrinsic clearance of tolbutamide, phenytoin, piroxicam and torsemide by CYP2C9*3. Preliminary results suggest that CYP2C9 alleles may have differing abilities to demethylate the pesticide methoxychlor. (Hu *et al.*, 2004).	
		CYP2C9*3 (and other mutant alleles)	Reduced activity		
CYP2C19	CYP2C19*1	CYP2C19*2 CYP2C19*3	Inactive enzyme	Prolonged sedation with diazepam.	
CYP2D6	CYP2D6*1	CYP2D6*2xn	Increased activity due to presence of multiple copies of the gene	Therapeutic failure of antidepressants in ultrarapid metabolisers.	
		CYP2D6*4 and other null alleles, collectively classified as CYP2D6*0	Inactive enzyme	CYP2D6*4 accounts for 70-90% of poor metabolisers, who have increased susceptibility to side effects of drugs e.g. debrisoquine, desipramine. Loss of the analgesic effects of codeine is observed clinically in poor metabolisers.	
CYP2E1	CYP2E1*1	*Pst*I and RsaI RFLPs in 5′ flanking region	Altered transcriptional regulation	Associated with susceptibility to lung cancer, nasopharyngeal carcinoma and alcoholic liver disease.	
		Coding variants	Few functionally significant changes	Activity is highly conserved, possibly due to role in gluconeogenesis during extreme fasting.	
CYP3A4	CYP3A4*1	CYP3A4*1B	Reduced steroid metabolism, but may just be acting as a marker for the presence of CYP3A5*1	CYP3A4*1B may be a risk factor for progression to prostate cancer in men with benign prostatic hyperplasia.	
		Many other variants, none individually detected at high frequency	Wide interindividual variation in activity mediated by genetic polymorphisms and differences in level of expression	Most variants do not alter activity dramatically. This leads to a broad unimodal distribution of activities, with a range of approximately 10-fold for *in vivo* clearance of alfentanil, midazolam. Trans activating factors such as PXR and CAR may play an important role in interindividual variability.	

Gene	Wild type	Common variants	Consequence of polymorphism	Comments	Recent review(s)
CYP3A5	CYP3A5*1	CYP3A5*3, CYP3A5*5, CYP3A5*6	Splicing defect	May be involved in susceptibility to the carcinogenic effects of aflatoxin B1; affects requirement for tacrolimus in transplantation patients.	
		CYP3A5*7	Frameshift mutation		
CYP3A7	CYP3A7*1A	CYP3A7*1B CYP3A7*1C	Altered transcriptional regulation	Originally thought to be exclusively a fetal form, but now known to be expressed in adult liver as well.	
Microsomal epoxide hydrolase		Tyr113His His139Arg	Reduces enzyme activity by 40% *in vitro*, possibly due to reduced protein stability	Increased chromosomal aberrations due to 1,3-butadiene exposure in individuals with low activity variants. Also associated with susceptibility to hepatocellular carcinoma, lung and ovarian cancer.	(Fretland and Omiecinski 2000; Lee *et al.*, 2002)
		5′-flanking region variants	Altered rates of transcription		
Aldehyde dehydro-genase	ALDH2*1	ALDH2*2	Reduced activity	Found only in Asian individuals. Reduced risk of alcoholism/alcohol abuse due to distressing effect of high peripheral blood alcohol levels, but increased risk of ethanol-related cancers in those who do drink. Possible association with increased DNA damage in workers exposed to vinyl chloride (Wong *et al.*, 1998).	(Vasiliou *et al.*, 2000; Quertemont and Tambour 2004)
NAT1	NAT1*4	NAT1*3 NAT1*10	High activity enzyme	May be associated with altered susceptibility to bladder cancer (aromatic amines), colon cancer (heterocyclic amines).	(Bruhn *et al.*, 1999)
		NAT1*11 NAT1*14	Reduced activity		
		NAT1*15	Inactive enzyme		
NAT2	NAT2*4	NAT2*5A NAT2*5B NAT2*5C NAT2*6 NAT2*7	Decreased enzymatic activity towards aromatic amine substrates	Increased susceptibility to isoniazid hepatotoxicity; increased incidence of bladder cancer.	(Hein *et al.*, 2000)

Gene	Wild type	Common variants	Consequence of polymorphism	Comments	Recent review(s)
GSTM	GSTM1*1	GSTM1*0	Gene deletion	Individual GST genes have only modest effects on specific tumour types, although homozygosity for a fully functional allele may confer protection. Presence of null GST alleles does increase susceptibility to diseases (such as asthma, allergies, atherosclerosis, rheumatoid arthritis and systemic sclerosis) with an inflammatory component.	(Eaton and Bammler 1999; Hayes *et al.*, 2005)
		GSTM1*1A GSTM1*1B	Point mutation		
GSTP	GSTP1*1	GSTP1*1A GSTP1*1B GSTP1*1C GSTP1*1D	One or more point mutations		
GSTT	GSTT1	GSTT1*0	Gene deletion		
UDPGT	TA(6)	(TA)5 (TA)7 (TA)8	Altered number of TA repeats in the promoter region leading to changes in gene regulation	Influences probability of developing adverse effects (diarrhoea) following use of irinotecan to treat colorectal cancer (Marcuello *et al.*, 2004).	(Wells *et al.*, 2004)
Paraoxonase	PON1	$PON1_{192Q/192R}$ $PON1_{55L/55M}$	Originally thought to affect enzyme activity *in vivo*, but this now shown to be due to use of non-physiological conditions for *in vitro* assays	Originally thought to affect enzyme activity in vivo, but this now shown to be due to use of non-physiological conditions for in vitro assays.	(Draganov and La Du 2004)
Sulphotrans-ferase	SULT1A1*1	SULT1A1*2 SULT1A1*3, SULT1A1*4	Reduced activity	Variants have theoretical ability to alter susceptibility to paracetamol toxicity, but this has not been demonstrated experimentally. Also hypothesised to impact on breast cancer, possibly via effects on 17β-estradiol metabolism, but not clear whether effect will be seen at physiological substrate concentrations. Effects on Tamoxifen therapy have been postulated but data are inconclusive.	(Glatt and Meinl 2004)
	SULT1A2*1	SULT1A2*2	Reduced activity		
NAD(P)H: quinone oxidore-ductase (NQO1)	NQO1*1	NQO1*2 NQO1*3	Reduced expression of NQO1 protein	Increased susceptibility to toxicity of benzene and quinone-based bioreductive drugs (e.g. mitomycin C).	(Phillips and Basu 2004)

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Appendix 3

Case study of methylmercury risk assessment

A3.1 In 2003 JECFA established a Provisional Tolerable Weekly Intake (PTWI) for methylmercury of 1.6 μg/kg bw/week. This was based primarily on recent prospective epidemiological studies of populations who eat large amounts of fish, and was set to protect against developmental neurotoxicity, considered to be the most sensitive toxicological endpoint of methylmercury exposure. Previously the PTWI had been established using human data from population poisoning incidents. However, there is also a range of experimental data available. A number of different data sets (e.g. animal studies versus human epidemiological data) have led to different PTWIs being derived.

A3.2 Toxicological data considered by JECFA and reviews by JECFA and other bodies, are drawn upon, together with papers in the peer-reviwed literature. The first section briefly summarises what is known about methylmercury and how knowledge of its toxicity has developed. This is followed by a brief description of the basis for the current JECFA PTWI established in 2003. This is then followed by sections which consider what PTWI would be indicated if based solely on the following data sets:

- Experimental data from animal and *in vitro* studies
- Experimental data from studies conducted prior to human poisoning incidents
- All data except epidemiological studies

Background

A3.3 Methylmercury is an organic form of mercury which is rapidly absorbed from the gastrointestinal tract. Approximately 95% of the methylmercury ingested is absorbed and peak blood methylmercury levels are reached within 6 hours. Methylmercury is able to cross plasma membranes more readily than inorganic mercury compounds, and readily crosses the blood-brain barrier and the placenta. Animal studies have shown that methylmercury is particularly able to enter the blood of the fetus, in which the blood-brain barrier is not fully developed. In the brain methylmercury accumulates and is slowly converted to inorganic mercury.

A3.4 Methylmercury is neurotoxic in humans and animals, though the mechanism by which this occurs is still unclear. Cases of neurotoxicity and some fatalities in humans have been reported since the late 19th century. However, the largest incidents by far were poisoning episodes in Japan and Iraq between the 1950s and 1970s, the former due to consumption of fish contaminated with methylmercury, the latter due to consumption of bread made with grain dressed with methylmercury as a fungicide.

A3.5 The first reported use of methylmercury in laboratory research was in 1863, which corresponds to the first reported human toxicity cases, severe disabling neurotoxicity progressing to fatality in two laboratory workers (Frankland and Duppa, 1863, cited in Hunter *et al.*, 1940). Methylmercury was first used as a fungicide for dressing seeds in 1914, and cases of neurotoxicity, with symptoms including severe generalised ataxia, dysarthria and gross constriction of the visual fields, were reported in workers involved in the manufacture of methylmercury for this purpose, apparently due to exposure via inhalation of methylmercury dust (Hunter *et al.*, 1940).

A3.6 In the 1950s the first cases of methylmercury poisoning in the general population were reported, usually due to the consumption of methylmercury-treated grain. In one case report (from 1952) methylmercury poisoning was reported in a 13-month old infant in Sweden, resulting from the use by a family of flour made from methylmercury-treated grain to prepare porridge (Engleson and Herner, 1952). The mother was asymptomatic, though mercury was identified in her urine; however, she subsequently gave birth to a baby girl who was severely affected. This appears to be the first piece of evidence that the developing nervous system of the fetus is more vulnerable to the effects of methylmercury. The symptoms in the baby girl included mental retardation, which was not seen in affected adults. The 13-month infant (exposed to methylmercury from about 9 months of age) also showed mental retardation, though not as marked.

A3.7 The poisoning incidents in Japan in the 1950s and 1960s through consumption of contaminated fish were the largest methylmercury poisoning incidents up to that time, and led to concern about mercury releases into the environment and ultimately exposure of the general population to lower levels of methylmercury through consuming fish from 'non-polluted' areas. The first incident was in and around the coastal Japanese town of Minamata in the 1950s. The residents of Minamata and surrounding villages relied largely on fish caught in Minamata bay as a protein source. In 1932 a Minamata company began making acetaldehyde, and inorganic mercury used as a catalyst in the production process began leaking into the bay. Some methylmercury was also produced in the process and released. In the early 1950s production boomed, but signs of neurotoxicity began to appear in residents. This had reached epidemic proportions by 1956. It was 1959 before methylmercury poisoning was diagnosed and linked to release of mercury into the bay. Close to 100 affected people had been identified by that time, more than 20 of whom had died. Although exposure to the methylmercury ceased, more affected people were identified over the succeeding years and children were born severely affected by the methylmercury, often to mothers who had shown only transient paresthesia. The total number of people affected by the incident is unclear, but more than 2000 people are officially recognised as having been affected. The neurological effects were sensory and motor impairment in exposed adults and mental retardation, cerebral palsy, deafness, blindness and dysarthria in individuals who had been exposed *in utero*.

A3.8 In the mid 1960s a similar outbreak developed along the Agano River in Niigata, Japan. As a result of the Minamata incident the effects of methylmercury poisoning were quickly recognised and public health measures taken to limit exposure (Myers *et al.*, 2004). Studies subsequent to these incidents consistently found elevated levels of methylmercury in fish from waters contaminated with mercury due to industrial processes.

A3.9 Mercury is widespread in the environment due to its uses in many products and industrial processes, and natural release from the Earth's crust. Mercury entering the environment, in whatever form, is converted into methylmercury by aquatic microorganisms. The methylmercury is then able to enter fish either directly from the water or by being concentrated up the food chain. Larger, older fish, particularly predatory species, contain the highest levels of methylmercury. Consumption of fish contaminated with methylmercury is the major source of exposure of the population to methylmercury (National Research Council, 2000).

A3.10 As well as neurotoxicity, methylmercury exposure has resulted in adverse effects on the kidneys in humans and animals. Effects on the liver, the reproductive and immune systems have been reported at

higher doses in animals. However, the most sensitive adverse effect, at least in humans, is considered to be developmental neurotoxicity. Some epidemiological studies have linked consumption of methylmercury-contaminated fish to cardiovascular disease, although results of case-control studies have been contradictory.

The basis for the JECFA PTWI

A3.11 Methylmercury was first considered by JECFA at its 16th meeting in 1972, and was subsequently considered at its 22nd, 33rd, 53rd and 61st meetings. A PTWI of 5 μg/kg bw/week was derived for mercury in 1972, with two thirds of the PTWI (3.3 μg/kg bw/week) allocated to methylmercury. This was retained in 1978. The PTWI was based primarily on human data from the Minamata and Niigata incidents of methylmercury poisoning through consuming contaminated fish. The lowest hair and blood mercury levels reported in adults with neurological impairment were converted to a steady state mercury intake from the diet to determine the PTWI. The conversion was based on data provided by several small human studies on the relationship between hair mercury content and mercury intake, and mercury level in blood cells and mercury intake. In 1989 JECFA noted that pregnant and nursing mothers were likely to be more susceptible to methylmercury but the available data were insufficient to recommend an intake specifically for those subgroups and the PTWI of 3.3 μg/kg bw/day for methylmercury was retained.

A3.12 The PTWI was again retained at the 53rd meeting in 2000, with JECFA noting that the fetus and infants may be at greater risk of toxicity. Data were available from epidemiological studies, including studies in the Faeroe Islands and the Seychelles; however JECFA concluded that these studies did not provide consistent evidence regarding effects on neurodevelopment in children of mothers with hair-mercury levels of 20 mg/kg and below. The committee recommended that the issue be reconsidered when data on 8-year neurodevelopmental evaluations of the Seychelles cohort were available.

A3.13 In 2003 methylmercury was evaluated again. Maternal hair mercury concentrations corresponding to a NOAEL from the Seychelles study and to a benchmark dose lower confidence limit (BMDL) for the Faeroe Islands study were calculated and averaged. This averaged hair concentration was converted to a steady state dietary methymercury intake using a one compartment pharmacokinetic model which used values appropriate for pregnancy. The resulting intake was divided by uncertainty factors of 2 and 3 to account for intra-individual variation in hair:blood ratios and the association between blood mercury concentration and intake. The resulting PTWI of 1.6 μg/kg bw/week was considered to be sufficient to protect the developing fetus as well as other groups of the population.

If all human data are excluded

A3.14 As described above, some human data on the toxicity of methylmercury have been available since 1863. In particular, a large amount of data has been provided by the tragic events in Minamata and Niigata in Japan and in Iraq, in which large numbers of people consumed methylmercury-treated grain in 1972, and these have been key to risk assessments by JECFA and other bodies. However, a considerable body of experimental data (animal and *in vitro* studies) is also available.

A3.15 Considering only the available animal and *in vitro* studies, a number of hazards associated with methylmercury are identified. These hazards are listed in Table A3.1, which summarises the lowest exposures associated with the adverse effects and any NOAELs. This table is not a comprehensive list of all studies on methylmercury.

Table A3.1. Summary of adverse effects of methylmercury identified in non-human toxicological studies, and lowest doses at which identified. Exposures are oral via the diet unless otherwise stated or *in vitro* studies

Adverse effect	Lowest doses associated with adverse effect	NOAEL	Reference
Decreased body weight	2 mg/kg bw/d i.p. for 13 days in male rats		Chuu *et al.*, 2001
	0.03 mg/kg bw/day in 52-month study in male monkeys	0.01 mg/kg bw/day	Kawasaki *et al.*, 1986
Neurotoxicity	0.1 mg/kg bw/day in 52-month study in male monkeys	0.03 mg/kg bw/day	Kawasaki *et al.*, 1986
Hepatotoxicity	8 mg/kg bw s.c. single dose in rats		Desnoyers and Chang, 1975
	3.2 mg/kg bw/day (estimated) for 11 months in cats		Chang and Yamaguchi, 1974
Renal toxicity	0.05 mg/kg bw/day and above in a 24 month study in Wistar rats	0.01 mg/kg bw/day	Munro *et al.*, 1980
Carcinogenicity	0.69 mg/kg bw/day in male but not female mice (renal tumours), suggested to be secondary to chronic nephropathy, observed in 47/60 animals at this dose	0.14 mg/kg bw/day	Mitsumori *et al.*, 1990
	–	2.5 mg/kg bw/day in 2 year study in rats	Verschuuren *et al.*, 1976
Reproductive and developmental toxicity (excluding developmental neurotoxicity)	10 mg/kg s.c. methylmercury chloride in male rats for 8 days decreased sperm production, testicular and prostatic lobe weights and plasma testosterone	0.05 mg/kg bwday	Homma-Takeda *et al.*, 2001
	2.5 and 5 mg/kg bw/day in female rats for 7 days reduced fertility and number of viable embryos per litter		Khera, 1973
	0.5 mg/kg bw/day for 90 days and 1 mg/kg bw/day for 35 days in female rats reduced number of viable offspring per litter		Khera, 1973
	0.25 mg/kg bw/day to Wistar rats from weaning resulted in eyelid lesions associated with hardening of lachrymal glands in offspring		Khera and Tabacova, 1973
	0.05, 0.07 or 0.09 mg/kg bw/day in macaque monkeys decreased number of viable deliveries where blood concentrations were above 1.5-2 μg/mL		Burbacher *et al.*, 1988
	0.05 and 0.07 mg/kg bw/day to male macaque monkeys resulted in abnormal sperm (bent tails) with decreased motility		Mohamed *et al.*, 1987
	Single s.c. doses of 1.6 mg/kg bw MeHg chloride on days 3, 5 or 9 of gestation increased numbers of malformations (clubfoot and hydrocephalus) in hamsters	1 mg/kg bw in pregnant female mice days 6-17 of gestation	Khera and Tabacova, 1973
			Harris *et al.*, 1972

Adverse effect	Lowest doses associated with adverse effect	NOAEL	Reference
Developmental neurotoxicity (prenatal or pre- and post-natal exposure)	Offspring of female macaque monkeys treated with 0.04 mg/kg bw/day MeHg by gavage for at least 198 days before mating demonstrated increased randomness in visual attention to novel stimuli when tested at days 5-60 of age	0.02 mg/kg bw/day (given on days 6-9 of gestation only)	Gunderson *et al.*, 1988
	Impaired spatial vision at 3 to 4 years of age and an increase in auditory threshold at 7 years of age in 5 infant macaque monkeys given 0.05 mg/kg bw/day MeHg chloride from 1 day after birth to 7 years of age. At 13 years of age signs of altered coordination and hesitance were observed in some animals and tests revealed a higher vibration threshold in 4/5 treated animals. At 20 years of age slight constriction in visual fields was identified in 2/5 monkeys		Rice and Gilbert, 1992; Rice, 1996; Rice and Hayward, 1999
	Offspring of dams given 0.04 mg/kg bw/day by gavage on days 6-9 of gestation showed increased passiveness and decreased habituation to an auditory startle at 60-210 days postnatally		Stoltenberg-Didinger and Markwort, 1990
	In vitro studies:		
	Axonal morphogenesis inhibited in primary cultures of chick forebrain neurons exposedto 62.7 or 125.5 μg/L MeHg		Heidemann *et al.*, 2001
	Reduced cell proliferation and death resulting from effects on signal pathways involved in cell cycle regeneration in primary rat CNS cells exposed to 0.25-1.51 mg/L MeHg		Faustman *et al.*, 2002
	Impaired cationic channel function in differentiating pheochromocytoma cells exposed to 0.025-7.53 μg/L MeHg or 6 days		Shafer *et al.*, 2002
	Inhibited cortical cysteine uptake in newborn Sprague Dawley astrocytes at 1.25-2.51 mg/L MeHg		Shanker *et al.*, 2001
Immunotoxicity	Estimated intake of approximately 0.59 mg/kg bw/day in mice for 12 weeks depressed natural killer cell activity and increased lymphoproliferation response to T- and B-cell mitogens		Ilbäck, 1991
	Offspring of BALB/C female mice exposed to estimated intakes of 0.075 or 0.75 mg/kg bw/day MeHg chloride in the diet for 10 weeks prior to mating and during gestation and lactation showed increased proliferative response to mitogens (0.075 mg/kg bw/day) and antigens (0.75 mg/kg bw/day)		Thuvander *et al.*, 1996

A3.16 Studies on genotoxicity have not been included in the table. Mixed results have been reported in *in vitro* studies. Studies in *S. typhimurium* strains TA98, TA97, TA100 and TA1537 with and without metabolic activation in rats and hamsters produced negative results for methylmercury chloride and methylmercury hydroxide (NTP, http://ntp.niehs.nih.gov, accessed 22/02/05). Methylmercury has been reported to cause DNA damage in *in vitro* studies in Bacillus subtilis, cultured human nerve and lung cells, Chinese hamster V-79 cells and rat glioblastoma cells, and chromosomal aberrations and aneuploidy in human lymphocytes (National Research Council, 2000).

A3.17 Methylmercury was negative in *Drosophila* sex-linked recessive lethal mutation studies *in vivo* (NTP, http://ntp.niehs.nih.gov, accessed 22/02/05). Single doses of 2.2, 4.4 or 8.9 mg/kg bw methylmercury chloride to male Swiss mice resulted in a dose-dependent increase in the frequency of chromosomal aberrations and percentage of aberrant cells in bone marrow. In a study in cats, doses of 0.0084, 0.02 or 0.046 mg/kg bw/day for 39 months were reported to produce a significant increase in the number of nuclear abnormalities in bone-marrow cells and to inhibit DNA repair, however, this was not dose related (National Research Council, 2000).

A3.18 From the data in Table A3.1, the most sensitive effects identified, excluding possibly neuro-developmental toxicity, for which there is no clear NOAEL, appear to be renal toxicity in Wistar rats and decreased body weight in monkeys. In both a 24-month chronic study in Wistar rats and a 52-month study in Rhesus and Mullata monkeys, the NOAEL was 0.01 mg/kg bw/day (Munro *et al.*, 1980; Kawasaki *et al.*, 1986). Clinical and histopathological signs of neurotoxicity occurred in monkeys at higher doses with a NOAEL of 0.03 mg/kg bw/day.

A3.19 The lowest dose associated with effects on neuro-development was 0.04 mg/kg bw/day in two studies: one in female macaque monkeys dosed for 198-747 days prior to mating, in which offspring showed increased randomness in visual attention to novel stimuli when tested at 50-60 days of age compared to controls (Gunderson *et al.*, 1988); and one in rats in which dams were treated with methylmercury on days 6-9 of gestation by gavage, and offspring exhibited increased passiveness and decreased habituation to an auditory startle at 60-210 days postnatally (Stoltenberg-Didinger and Markwort, 1990). A NOAEL of 0.02 mg/kg bw/day is available from the Stoltenberg-Didinger and Markwort (1990) study in rats. However, the methylmercury was only given for 4 days of gestation. It is therefore not clear whether this NOAEL is appropriate.

A3.20 No NOAEL is available from studies on immunomodulation, however these have generally involved higher doses. Relatively minor effects (increased lymphoproliferative response to B and T cell mitogens) were apparent in offspring of mice treated with 0.075 mg/kg bw/day for 10 weeks prior to mating, through gestation and lactation.

A3.21 If the lowest reported NOAEL of 0.01 mg/kg bw/day, from chronic oral feeding studies in both rats and monkeys, was used to determine a PTWI, and a total uncertainty factor of 100 was applied, the resulting PTWI would be 0.7 μg/kg bw/week. This is lower than the PTWI derived by JECFA using human data (1.6 μg/kg bw/week).

A3.22 It is possible that an extra uncertainty factor would be applied given that no appropriate NOAEL is available for neuro-developmental toxicity. However, the lowest dose associated with adverse neuro-developmental effects was 4 times the NOAEL used to derive this PTWI.

Extrapolation between species on a body surface area basis

A3.23 It should be noted that JECFA observed that extrapolation between species on a body weight basis gave nonsense values (for example doses of more than 1 mg/kg bw/day were frequently used in rodent studies with minimal effects, whereas this was in the range of intakes associated with severe neurotoxicity in a mass human poisoning outbreak in Iraq). JECFA noted that clearance half-lives for methylmercury increased with the mass of the animal species and considered that allometric extrapolation based on body surface area could be used to extrapolate between species. This was not applied above since all human data, including pharmacokinetic data, were excluded and thus the inappropriateness of extrapolating between species on a body weight basis may not have been identified.

A3.24 If scaling on a body surface area (body weight$^{0.75}$) basis were used, and bodyweights of 400 g for a rat and 5 kg for a monkey assumed:

- the NOAEL of 0.01 mg/kg bw/day from the chronic rat study would be equivalent to a dose of approximately 0.108 mg in a 70 kg human, or 0.0015 mg/kg bw/day.
- The NOAEL of 0.01 mg/kg bw/day from the chronic monkey study would be equivalent to 0.36 mg in a 70 kg human, or 0.0052 mg/kg bw/day.

A3.25 If a total uncertainty factor of 100 was applied this would result in a PTWI of 0.11 μg/kg bw/week from the study in rats or 0.36 μg/kg bw/week from the study in monkeys. If the factor of $10^{0.6}$ for interspecies differences in toxicokinetics was removed from the uncertainty factor, resulting in an uncertainty factor of 25, the PTWI based on the rat study would be 0.42 μg/kg bw/week (or based on the monkey study, 1.5 μg/kg bw/week).

Experimental data prior to the Minamata incident in Japan

A3.26 Attempts to identify experimental toxicological studies prior to human cases of methylmercury poisoning have been unsuccessful. The scientific literature contains references to human case reports of poisoning with methylmercury and other organic mercury compounds as early as 1863 (Hunter *et al.*, 1940; Swensson, 1952), with clearly described symptoms of neurotoxicity. However, the earliest identified report of experimental toxicological studies is the paper of Hunter *et al.* (1940). This section will therefore focus on experimental data available before 1959, i.e. prior to recognition of methylmercury poisoning in Minamata, Japan, the first mass outbreak of methylmercury poisoning.

A3.27 Hunter *et al.* (1940) described four case studies of methylmercury toxicity in individuals employed in the manufacturer of methylmercury seed dressings and also reported four animal experiments undertaken to determine whether symptoms similar to those observed in the four patients could be reproduced in

animals and to provide material for histopathological study. These experiments are summarised in Table A3.2.

A3.28 Histopathological analysis of affected rats indicated that methylmercury caused, in the first instance, a severe Wallerian degeneration in the peripheral nerves, posterior spinal roots and trigeminal nerve, followed by degenerations in the posterior columns and the descending root of the trigeminal nerve. The authors concluded that the clinical course taken by the experimental animals followed so close a parallel to that shown in human poisoning cases that the pathological basis of the condition was probably parallel to both.

Table A3.2. Summary of experimental studies performed by Hunter *et al.* (1940):

Species/strain	Exposure	Observed effects	NOAEL
Norwegian rats	0, 36 mg/kg bw/day methylmercury iodide (3 rats) or 34 mg/kg bw/day methylmercury nitrate (2 rats) for 29 days p.o. by gavage	No symptoms for 2 weeks, then rapid loss of weight. Symptoms of neurotoxicityobserved in 4th week, including progressive ataxia of hind legs. All animals died within 11 days of onset of symptoms	None
Norwegian rats	Four animals exposed to air 'laden' with methylmercury iodide vapour for 8 hours/day	Red eyes and nasal discharge, dazed appearance at times, attacks of hiccup after 10 days, irritability after 15 days, severe ataxia developed after 19 days. All animals died.	None
Norwegian rats	As above except for 'shorter periods' (approximately 4.6 hours/day). Two animals removed on the 8th day (37 hours exposure), one removed on the 14th day (64 hours total exposure), 7 animals exposed for 16 days (71 hours total exposure).	Two animals removed after 37 hours showed no clinical symptoms other than weight loss which was reversible. Animal removed on 14th day showed ataxia of hind legs, this progressed after removal from exposure until found moribund on day 36. One exposed for 16 days showed symptoms of neurotoxicity 9 days after exposure which did not reverse. Of remaining 6 animals, four showed some mild clumsiness of hind legs which did not progress, the remainders were unaffected other then temporary weight loss.	None
Rhesus monkey	1 adult female monkey exposed to methylmercury iodide vapour for daily periods increasing from 1 to 7.5 hours to give a total exposure period of 71 hours in 21 days.	Weight loss, subdued and 'bedraggled' appearance towards end of study, became irritable, weak in the hind legs and clumsy in movement. Progressed after cessation of exposure to become severely ataxic, with forelimbs affected in addition to hind legs.	None

A3.29 Lundgren and Swensson (1950) reported the probable lethal dose for methylmercury toluol sulphonate administered by single intraperitoneal injection to mice to be 27.5 mg/kg bw. Swensson (1952) reported a series of acute and subacute studies on methylmercury in which the methylmercury was adminstered intraperitoneally or intravenously. These are summarised in Table A3.3.

Table A3.3. Summary of acute and subacute studies with methylmercury compounds (Swensson, 1952)

Type of study	Species/strain	Exposure details	Outcome
Acute	Mouse	? i.p. as methylmercury chloride	LD50 15.5 mg/kg bw/day
Acute	Rat	? i.p, presumably as methylmercury chloride	Probable Lethal Dose "about" 10 mg/kg bw
Acute	Rabbit	4, 8, 16 mg/kg bw i.v. – one animal per group	Animals dosed 4 and 8 mg/kg bw survived without clinical symptoms, animal dosed 16 mg/kg bw died within 24 hours
Subacute	Rat	? i.p. every other day for 4 weeks	Clinical effects including signs of "nerve damage" observed but doses not clear

A3.30 Some histopathological examinations of the central nervous systems of animals used in the above studies were undertaken. These were reported to show "cell injuries" in the granular layer of the cerebellum, in the Purkinje cells in the cerebellum and in the cells of the spinal cord.

A3.31 No other experimental data prior to 1959 have been identified. From the limited animal data available, therefore, neurotoxicity was apparent as a key adverse effect. However, the studies were undertaken in response to cases of human toxicity, and no NOAELs are available from these studies for risk assessment. A PTWI could not therefore be derived from these data.

Including all data except the recent epidemiological studies

A3.32 The former JECFA PTWI of 3.3 μg/kg bw/week was established prior to epidemiological studies being conducted in high fish-eating populations in New Zealand, the Faeroe Islands and the Seychelles. This section will re-examine the data which were used to determine the former PTWI.

A3.33 Excluding epidemiological studies, a relatively large amount of human data is still available, primarily from the large poisoning outbreaks from methylmercury contamination of fish in Minamata and Niigata, and from Iraq in 1972 in which people were affected by methylmercury through using grain intended for planting, which had been treated with methylmercury, to bake bread.

A3.34 In its 1972 evaluation, JECFA used published studies on methylmercury-intoxicated patients from the Niigata area to identify the lowest hair and blood methylmercury concentrations at time of exposure associated with symptoms.

A3.35 The lowest blood and hair mercury concentrations in Niigata patients were identified. From small studies the half-lives of mercury in hair and blood were also identified. These were 50-108 days (median 66 days) in the hair of 8 patients, and 35-137 days (median 55 days) in blood of 7 patients. Using these half-lives the hair and blood mercury concentrations were extrapolated back to those at onset of symptoms. JECFA noted that extrapolated hair concentrations in Niigata patients were usually between 200 and 1000 μg/g, but in one case the concentration was 50 μg/g. Extrapolated blood mercury concentrations were between 0.2 and 2 μg/g, equivalent to concentrations in blood cells of 0.4 to 4 μg/g. The lowest

hair and blood mercury levels were associated with paraesthesia in some adults, although there were others with higher levels who showed no evidence of methylmercury toxicity.

A3.36 JECFA used data from other studies which had investigated the relationship between mercury intake and mercury concentration in hair and blood cells to convert the lowest blood and hair mercury concentrations to daily mercury intakes. These corresponded to 3 mg/day Hg, which could be considered a LOAEL. JECFA apparently used an uncertainty factor of 7 to derive a PTWI for mercury of 5 μg/kg bw/week. JECFA stated that two thirds of the PTWI, i.e. 3.3 μg/kg bw/week, should be applied to methylmercury.

A3.37 The PTWI of 3.3 μg/kg bw/week was maintained by JECFA as recently as 2000. Although JECFA noted that the fetus and infants may be at greater risk of toxicity the available data were not considered adequate to derive a PTWI that would be protective for these groups, pending the completion of analyses from an epidemiological study in the Seychelles.

A3.38 Data from the Niigata incident were used as methylmercury poisoning was quickly identified in the Niigata area since the symptoms were widely known in Japan as a result of the previous Minamata incident. Hair and blood mercury levels were therefore analysed more quickly in Niigata patients, enabling a more accurate extrapolation of mercury levels to time of exposure. In Minamata patients it was difficult to draw conclusions regarding exposure levels at time of exposure as measures of exposure were only taken years after the poisoning.

A3.39 The incident in Iraq resulted in approximately 600 deaths and at least 6000 cases of methylmercury poisoning; exposure, through consumption of methylmercury-treated seed grain, was more acute and higher dose than that in Japan. Hair mercury samples were taken from exposed pregnant women and a cohort of 81 children exposed *in utero* identified. The children were subsequently followed up and assessments of their neurodevelopment made (Marsh *et al.*, 1987). The data from this cohort have been re-analysed on various occasions. As these studies are epidemiological in nature they will not be considered further here.

A3.40 It can be concluded that, if epidemiological studies are excluded, the developing central nervous systems of the fetus and infants can still be identified as being particularly susceptible to methylmercury from the remaining human data from poisoning cases. However, it would not be possible to identify a PTWI protective of these groups without prospective studies measuring exposure in pregnant mothers and subsequently monitoring and assessing the children.

Evaluation of the 2003 JECFA PTWI

A3.41 A recently-published study provides some further validation of the 2003 JECFA PTWI. Blood mercury levels were measured in 474 older people aged 50-70 years and the subjects were assessed in 12 different neurobehavioural tests (Weil *et al.*, 2005). The hypothesis behind the study was that older people are at increased risk of cognitive decline and may be more sensitive to the effects of methylmercury.

A3.42 Analysed by multiple linear regression with adjustment for confounding factors, increasing blood mercury levels were associated with statistically significantly worse performance in one test (Rey complex figure delayed recall, a test of visual memory) and better in another (finger tapping, a test of motor and manual dexterity). There were no differences in the other tests. The authors concluded that due to a large number of multiple comparisons, and because the differences seen were in different directions, it could not be excluded that these results were due to chance. The authors concluded that the study provided no compelling evidence that blood mercury levels in this group were adversely associated with neurobehavioural performance.

A3.43 Blood mercury levels ranged from 0-16 μg/L in this study. The median was 2.1 μg/L. Using the model used by JECFA in 2003, this would correspond to a steady state intake of 0.4 μg/kg bw/week, which is within the 2003 PTWI. The highest measured blood mercury level would correspond to a steady state intake of 3.0 μg/kg bw/wk. This exceeds the 2003 PTWI, but is within the previous JECFA PTWI, which was considered protective against non-developmental adverse effects.

Conclusions

A3.44 The exercise excluding all human data enables a comparison between the PTWI based on human data and the PTWI that may result using experimental data, and thus enables some validation of the uncertainty factors used in extrapolating from animal studies to the general human population.

A3.45 The animal data indicate that there are significant interspecies differences for methylmercury, particularly in toxicokinetics. There are also some inadequacies in the database, for example in developmental toxicity. However, the resulting PTWI, derived using a total default uncertainty factor of 100, was within the 2003 JECFA PTWI derived using human epidemiological data, indicating that the total uncertainty factor was sufficient to account for the interspecies differences and data limitations, as well as inter-individual differences for methylmercury. This exercise therefore provides support for the default 100-fold uncertainty factor.

References

The references to this appendix are included in the main Reference section (Page 165)

Appendix 4

Whole-body Physiologically Based Pharmacokinetic Modelling (PBPK)

A4.1 Whole-body physiologically based pharmacokinetic modelling (PBPK), a mathematical model is constructed that can be used to predict the likely tissue levels of a chemical with time in humans based on animal data and *in vitro* data and scaling this information using human physiological parameters. Model predictions can then be compared with available experimental data, and the model parameters adjusted as necessary to minimise the discrepancy between predictions and observations. The model must include the critical organ or organs as specific components and some knowledge of concentrations eliciting adverse effects in these tissues is critical to the application of any model to risk assessment. Aspects of the predictability of the model in humans can be determined experimentally for pharmaceuticals but this is not possible for most other chemicals and there will be uncertainty arising from this limited validation. PBPK modelling aids in reducing uncertainty regarding exposure and in itself PBPK does not reveal anything about variations in susceptibility of a target tissue to exposure (toxicodynamics). If the mechanism of action is known however and data exist on the variability in the response with exposure within the target tissue, then the toxicodynamic (biological response) component can be characterised and modelled. The response model may contain various sources of uncertainty; these will include any extrapolation of the response effect profile from isolated targets (receptors, enzymes, etc.) to the *in vivo* situation together with available knowledge on the variability and distribution of the target within the population. This toxicodynamic (biological response) model can be combined with a PBPK model to provide exposure effect data, which would decrease the overall uncertainty in scaling data to humans. The sources of uncertainty in the integrated model will be the combination of those in the individual models.

A4.2 Whole-body physiologically based pharmacokinetic/toxicokinetic (PBPK/ PBTK) models (Nestorov, 2003; Rowland *et al.*, 2004) are of a fundamentally different nature. Here the philosophy is to overlay compound specific data, such as metabolic enzymatic activity and lipid solubility, onto an essentially independent model structure, representing biologically meaningful and independently verifiable physiological characteristics such as organ blood flow and tissue size. An attractive feature of these models is that their structure is essentially common to all mammalian species, thereby facilitating interspecies scaling. In addition, as relevant physiological and morphological data becomes available, as well as knowledge as to how compounds interact with the components of the system, the possibility exists for efficient use of limited compound-specific data in order to make reasonably accurate predictions as to the temporal profile of specific compounds, both within and between species, as well as under a variety of conditions (Poulin and Theil, 2002; Rodgers *et al.*, in press).

A4.3 As long ago as 1937, Teorell (Teorell, 1937) developed equations which describe the behaviour of foreign compounds in animal tissues. At the time such complex equations were essentially insoluble and his models were discarded for simpler compartmental representations. With the advent of powerful digital computers interest in such models rekindled in the 1960s and increasingly sophisticated physiologically based models are now being investigated (Nestorov, 2003). In these PBPK/PBTK models each organ or tissue is represented by a differential equation based on mass balance of xenobiotic entering, leaving and residing in that organ (Figure A4.1). This takes into account organ mass, organ blood flow, plasma protein binding, tissue affinity, membrane permeability, as well as enzymatic and transporter activity. Non-linearities are explained by features such as saturable metabolism or transport, enzyme induction, and changes in organ blood flow secondary to pharmacological or toxic effects (Stenner *et al.*, 1997). More recently a range of different tissue models have been invoked to explain subtleties of spatial interaction between xenobiotic, eliminating tissue, and perfusing blood (Rommel *et al.*, 1998). An equation estimating

rate of change of toxicant concentration in arterial blood incorporates the rates of change of efflux from all other organs representing the circulatory nature of blood flow (Fig A4.2). Numerical integration of these differential equations allows prediction of the time course of toxicant in each individual tissue. Physiological and physicochemical parameter values are usually obtained from the literature or from independent in-vitro experimentation. Predictions can subsequently be compared with observed concentration time data to confirm model structure. In practice, some parameters may be unknown and have to be estimated by fitting the model to observed concentration time data (Smith and Evans, 1995). A Bayesian approach may be used to incorporate information from both literature derived parameter values and from observed concentration time data to give both prediction intervals for tissue concentrations rather than simply a mean value (Bernillon and Bois, 2000). The prediction intervals incorporate elements of biological variability and of methodological uncertainty. Where discrepancy between observed and predicted concentration measurements cannot be explained by biologically plausible parameter values the model structure may come into question. It is common for there to be species differences in route of metabolism and accounting for these often improves agreement between observed and model predicted points (Lilly *et al.*, 1999).

A4.4 If they are to be of more than academic interest PBPK/PBTK models require some form of validation (Edler *et al.*, 2002). Models in which all structural parameter values are obtained independent of model building have some face validity, and external validation is provided by agreement between predicted and observed tissue concentration values. However, unlike the statistical procedures for fitting sums of exponential terms to data, the concept of "agreement" is qualitative with no consensus as to what defines acceptable goodness of fit. As yet there has been little consideration of model misspecification, what degree of disagreement between predicted and observed data indicates this and how structural uncertainty might be incorporated into the error bounds of parameter values and predicted tissue concentrations (Tucker and Ferson, 2003). Using the model for data analysis to estimate parameter values ideally requires an independent set of concentration-time data to provide validation (WHO, 2000-2001). Physiologically based models have large numbers of independent parameters some of which may be redundant in determining blood concentration time data but influential for local tissue or metabolite concentrations (Sweeney *et al.*, 2003). Theoretically at least, a model validated against blood concentration-time data alone might predict spurious local tissue concentrations. In practice models are validated by testing predicted concentration-time profiles against experimental data following a wide range of inputs and physiological conditions, as well as, for extensively metabolised compounds, measuring as many metabolite concentrations in as many tissues as possible (Andersen, 2003). In addition, insight into the relative influence of parameters on predictions can be gained through local and global sensitivity analysis. However, it is also important to take samples for analysis at appropriate times that allow the best chance of parameter estimation and testing of the model. PBPK/ PBTK models developed in animals can be used to predict tissue toxicant concentrations in humans by utilising the appropriate human physiological values. However, aspects that need to be kept in mind when extrapolating from animals to man, are going from high dose in animals to low dose in man, potential differences in route of administration, and differences in time scale of exposure. If there are known differences in metabolic pathways or specific transport mechanisms some adjustment to the structure of the model will also be required. When PBPK modelling is used in drug development there is the luxury of validating the extrapolated model against observations in humans at least for those compounds that continue into clinical phases (Levitt, 2002). In toxicology there is rarely the opportunity to have experimental data in

man but model predictions can be triangulated with observational data, such as those from sporadic cases of toxicity or concentration measurements obtained from individuals exposed to sub-toxic doses (Jonnson *et al.*, 2001).

A4.5 A unique advantage of whole body PBPK/PBTK models is their facility to incorporate even hazy information about variability and uncertainty into predictions of tissue concentrations and hence toxicity (Nestorov *et al.*, 2002). There is also the potential to investigate the impact of physiological variability and how this is determined by age, sex, ethnicity, genetics and disease and how these effects propagate through apparently complex models (Gueorguieva *et al.*, 2004).

A4.6 An attractive use of PBTK models is in the hazard characterization of essential nutrients such as vitamin A or that are toxic at higher doses. Here intakes associated with toxicity are often little greater than that associated with deficiency states (Dybing *et al.*, 2002) and an ultra-precautionary approach might result in malnutrition. The in-built conservatism of hazard characterization based on uncertainty factors is inappropriate as human tolerable intakes calculated from animal data may be less than reference doses required to avoid deficiency (Renwick *et al.*, 2003). Even the normal uncertainty factor allowed to protect vulnerable subgroups within the human population is often abandoned (United States Environmental Protection Agency, 2004a). A PBTK model has the potential to explore differences in tissue kinetics and help identify subgroups in whom the risk of toxicity exceeds that of deficiency. A PBTK model has been proposed for manganese to explore the apparent discrepancy in approach to oral and inhaled exposures with suggested uncertainty factors of 1 for the former and 1000 for the latter (Andersen, 2004). Although not yet complete it demonstrates just how complex some models may need to be to satisfactorily explain the full response to a toxicant.

Retinoic acid

A4.7 The example of retinoic acid teratogenicity illustrates the steps in using a PBPK/PBTK model for hazard characterization. Tretinoin is a potent teratogen in man (Lammer, 1985) but has therapeutic potential in the treatment of leukaemia (Warrell *et al.*, 1993), acne, and photo-aging (Griffiths *et al.*, 1993). Sufficient drug is absorbed when small doses are applied topically for there to be justifiable concerns about possible teratogenicity. At the request of the FDA a PBPK model was used to help clarify this risk before approval was given for its use to treat photo aging (Clewell, 1997). The initial model was developed in rats and included components to represent gut absorption, metabolism and placental transfer (Figure A4.3). As the behaviour of many organs were not of specific individual interest they were lumped together as "richly perfused" and "slowly perfused" groups and attributed common physiological parameters. As usual, each tissue was represented by a differential equation derived from mass-balance considerations with physiological values obtained from the literature. Refinements to the base model included enterohepatic recirculation and saturable hepatic metabolism described by the Michaelis-Menten equation. The model adequately described observed plasma tretinoin concentrations after intravenous administration of 3 different doses in rats (Figure A4.4). The initial model was subsequently transposed to the monkey with appropriate recognition of the similarities in tissue distribution but differences in metabolism of tretinoin between rodents and primates, oxidation as opposed to glucuronidation predominating in the latter. Again the model provided an adequate prediction of observed concentration after intravenous tretinoin dosing (Figure A4.5). As tretinoin is used in the treatment of leukaemia it was possible to obtain some human drug

concentration-time data following oral dosing to confirm the adequacy of the human version of the model incorporating zero-order gastric emptying and first-order uptake of drug from the intestinal lumen (Figure A4.6). In the treatment of photo-aging tretinoin is applied topically and a dermal absorption model with diffusion limited equilibration between formulation and stratum corneum and subsequently viable epidermis was added to the earlier base model. Parameters describing dermal uptake were estimated from in-vitro experiments with human cadaver skin. Predicted and observed total radioactivity after topical application of 100mg tritiated tretinoin to non-pregnant volunteers is shown in Figure A3.7. For the assessment of teratogenic potential it was necessary to assume an appropriate tissue dose metric, in particular the chemical species responsible for teratogenicity and the appropriate measure of exposure best linked to teratogenicity. Comparing measured concentrations of parent drug and metabolites and different measures of fetal exposure across mouse, rat and monkey each receiving the minimal teratogenic dose the Cmax of total active retinoids varied least and was therefore assumed to be the appropriate species and metric. The full model predicted a Cmax of total active retinoids in human fetus following extensive topical application by pregnant mothers some 5 orders of magnitude less than that associated with teratogenicity observed in animals, and tretinoin is now approved for human use.

Limits to the application of PBPK/PBTK

A4.8 PBPK/PBTK models are data intensive: despite the power and mechanistic appeal of PBPK/PBTK models there are several practical issues that have limited their wide application, and which need to be addressed. Implicit in the application of these models is the need for verifiable and constantly updated physiological and biochemical data bases for both animals and human. While much such data exist for the more common animals studied, pertinent data are often lacking , and comprehensive, easily accessible, data bases are still not readily available. Furthermore, while it is common to find mean data, information about the underlying variability in physiological parameter values is less available, and covariance estimates almost never. Sometimes there are differences in quoted values due to the various methods employed in their estimation; this is particularly true of tissue blood flow. Also, the software for both estimation and simulation, of which there is a variety is generally more complex and sophisticated than that used for characterising empirical models, such as the sum of exponentials, and in turn requires the user to be generally an experienced modeller. The number of researchers with the necessary skills is currently limited, and training of more researchers in the area is needed (Rowland *et al.*, 2004).

A4.9 PBPK/ PBTK models are valuable tools for extrapolating findings in animals to human and are an accepted part of hazard characterization (Aarons, 1999). Model building and essential validation can involve a great deal of work. Using these models raises issues that are dealt with implicitly in other methods of hazard characterization. These include knowledge of the proximate site of action, the critical chemical species, the appropriate tissue dose metric, and the dose metric response relationship. Nevertheless PBPK/PBTK modelling increases understanding of the mechanisms of toxicity and in some cases the results obtained are sufficient to form the basis of hazard characterization (United States Environmental Protection Agency, 2004b).

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Figure A4.1 Sub-model for toxicant mass balance in individual tissue

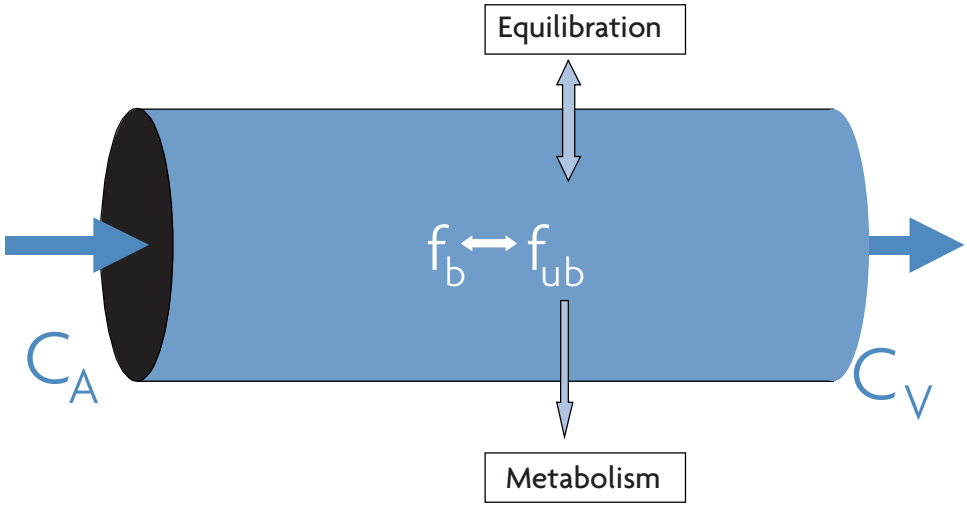


Figure A4.2 Base PBPK model

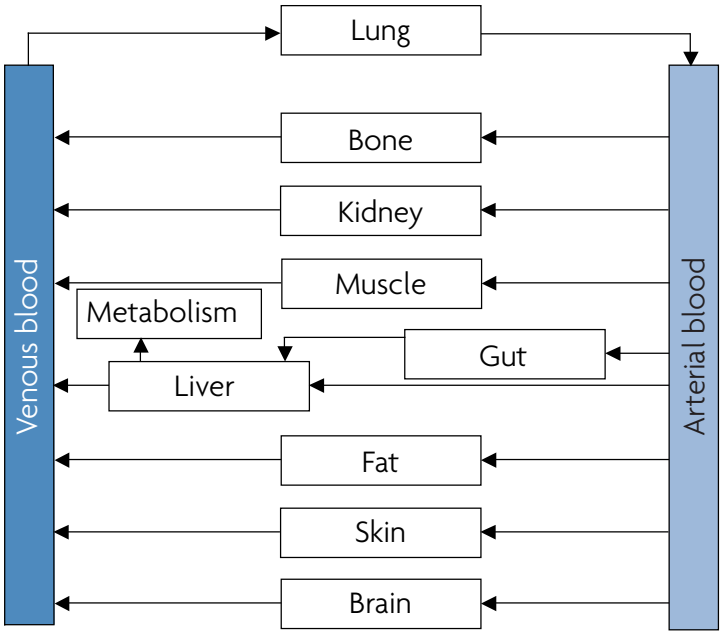


Figure A4.3 PBPK model for all-*trans*-retinioic acid (tretinoin).

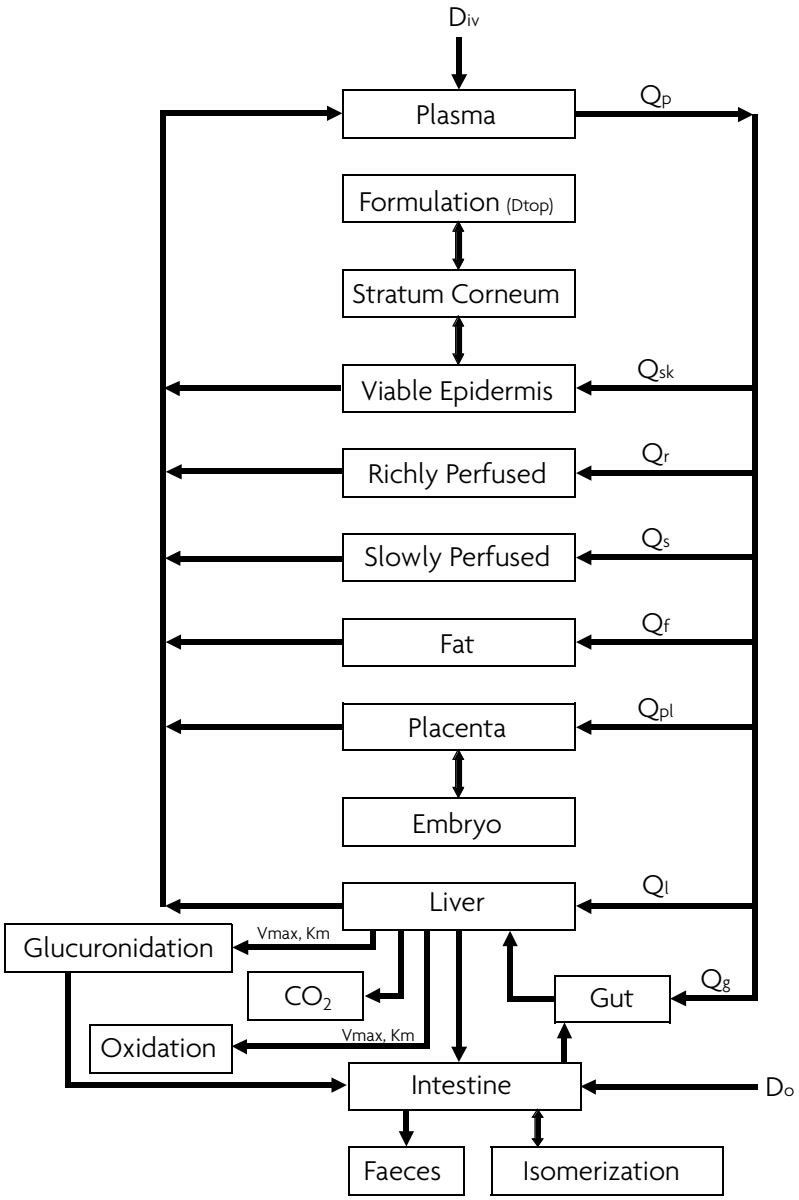


Figure A4.4 Observed (points) and model-predicted (curves) mean plasma tretinoin concentrations (nanograms per millilitre) after intravenous administration of tretinoin to rats. + 0.015mg/kg; ▲, 0.25mg/kg; o, 5.0mg/kg

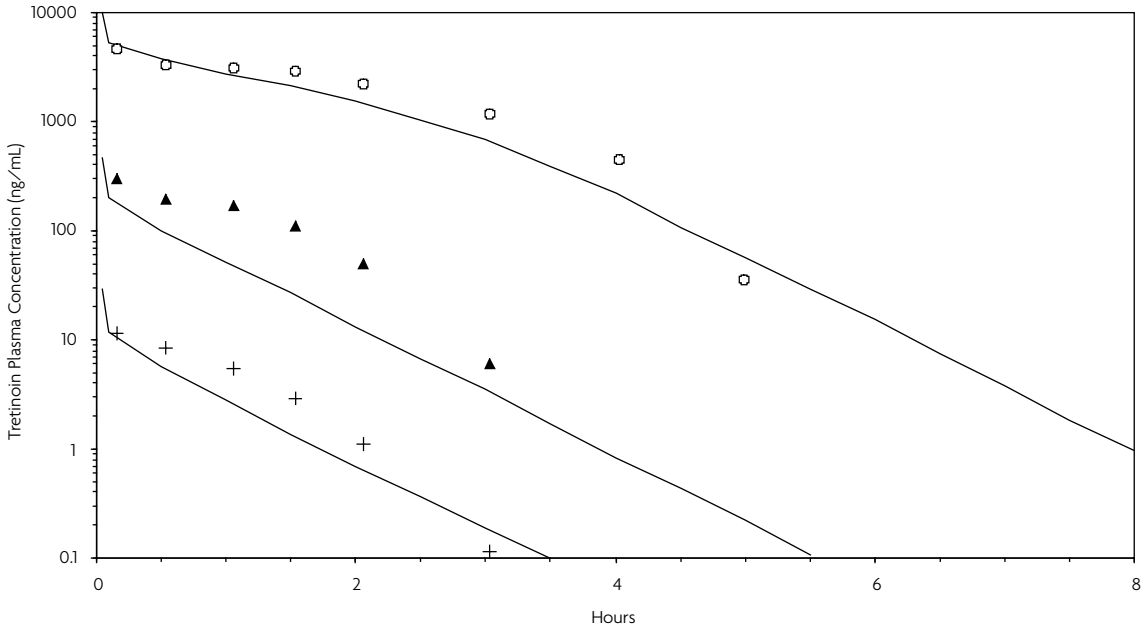


Figure A4.5 Observed (points) and model-predicted (curves) mean plasma tretinoin concentrations (nanograms per millilitre) after intravenous administration of tretinoin to monkeys. Plus 20mg/m²; triangle, 50mg/m²; circle, 100mg/m²

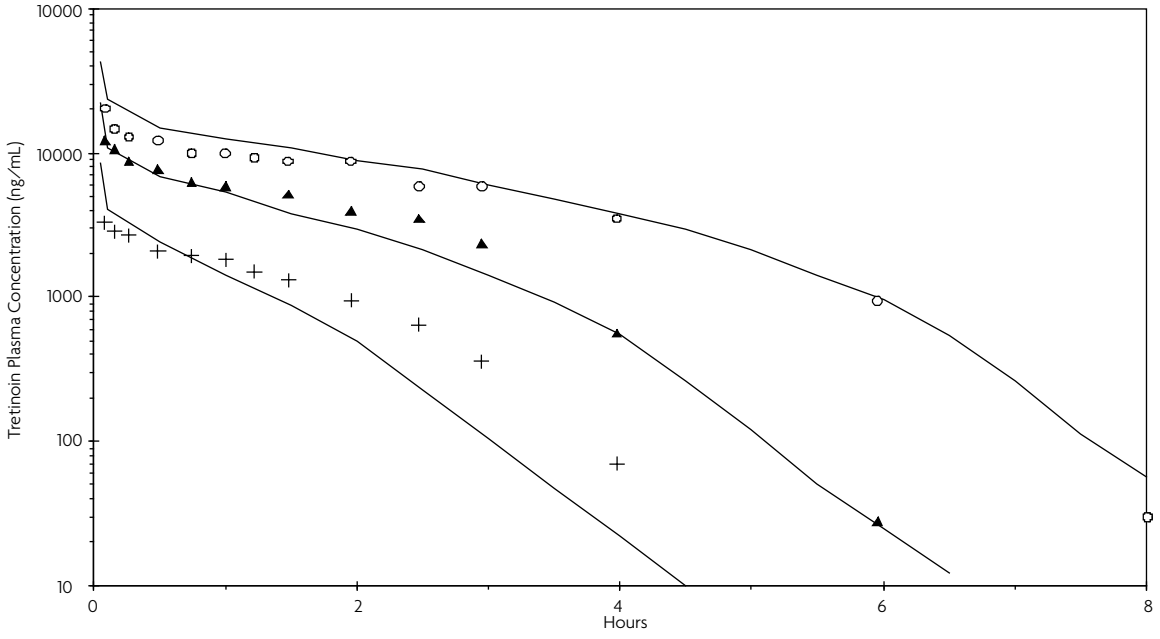


Figure A4.6 Observed (points) and model-predicted (curves) mean plasma tretinoin concentrations (nanograms per millilitre) after oral administration of 1.1mg/kg tretinoin to human beings

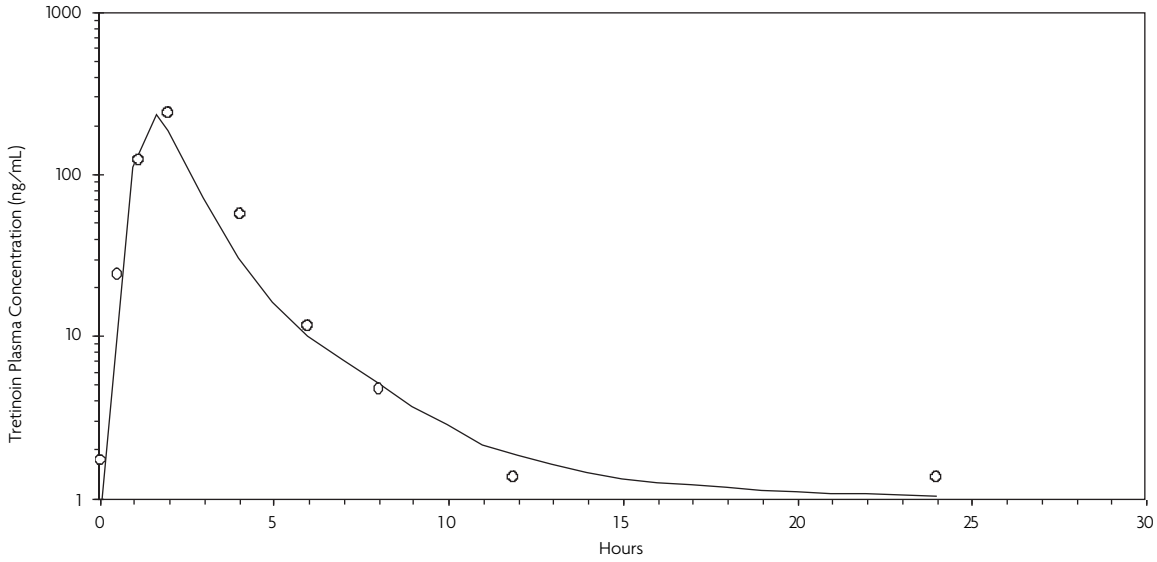
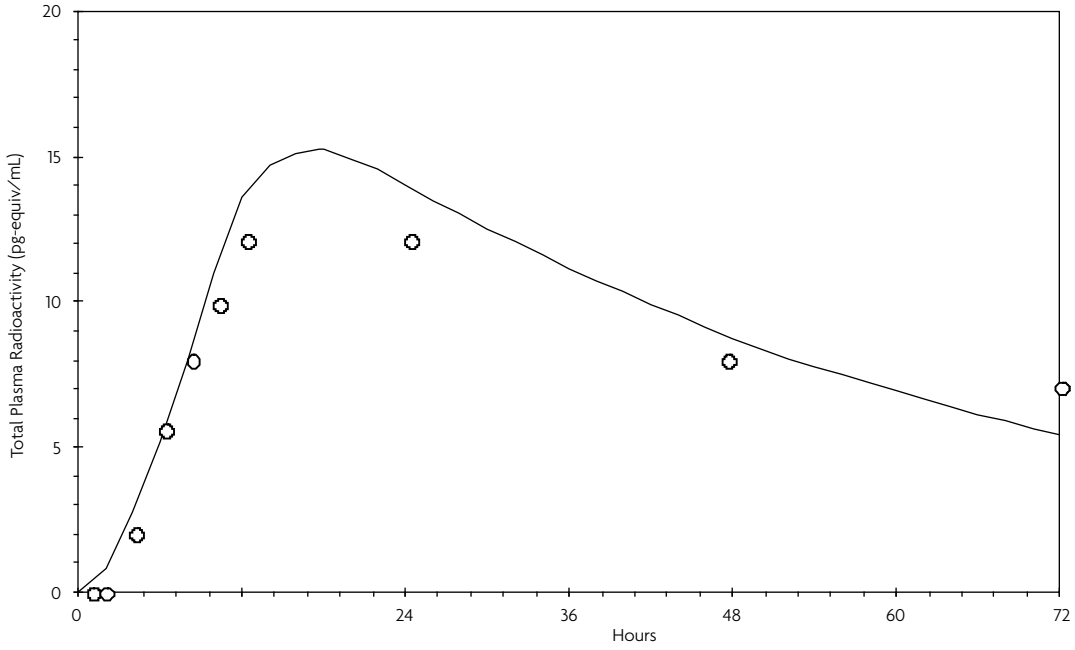


Figure A4.7 Observed (points) and model-predicted (curves) mean total plasma radioactivity concentrations (pictogram equivalents per millilitre) after topical application of 100mg tritiated tretinoin to human beings



Appendix 5

Abbreviations, acronyms and definitions

ADH	Alcohol dehydrogenase, an enzyme involved in the oxidation of ethanol
ADHD	Attention deficit hyperactivity disorder
ADI	Acceptable daily intake. Estimate of the amount of a substance in food or drink, expressed aon a body weight basis, that can be ingested in a period of 24 hours or less without appreciable health risk.
ADUF	Animal to human toxicodynamic adjustment factor
AKUF	Animal to human toxicokinetic adjustment factor
ALARP	As low as reasonably practicable: with compounds for which no threshold can be set for toxic effects, the risk assessment advice is that exposure should be as low as reasonably practicable
ALDH	Aldehyde dehydrogenase, an enzyme involved in the oxidation of *inter alia* acetaldehyde
AhR	Aryl hydrocarbon receptor (Ah-receptor). The Ah receptor protein regulates some specific gene expressions associated with toxicity. The identity of the natural endogenous chemicals which bind to the Ah-receptor is unknown. Binding to the Ah-receptor is an integral part of the toxicological mechanism of a range of chemicals, such as chlorinated dibenzodioxins and polychlorinated biphenyls.
AhRR	Ah-receptor repressor
Aleatory uncertainty	Synonym for variability as used in this report. Aleatory means "pertaining to luck", and derives from the Latin word alea, the rolling die.
ARfD	Acute referance dose. Estimate of the amount of a substance in food or drink expressed on a body weight basis, that can be ingested in aperiod of 24 hours or less without appreciable health risk.
ARNT	Aryl hydrocarbon receptor (AhR) nuclear translocator
AUC	A term used in pharmacokinetics for the area under the concentration-time curve for the clearance of a substance from a compartment (usually the blood).
Bayesian statistical methods	Statistical methods based on Bayes' theorem (named after British mathematician Thomas Bayes [1702-1761]). Bayes' theorem is a result in probability theory, which relates the conditional and marginal probability distributions of random variables. In some interpretations of probability, Bayes' theorem tells how to update or revise beliefs in light of new evidence.

BCRP	Breast cancer resistance protein.
Benchmark dose (BMD) modelling	An approach to dose-response assessment that aims to be more quantitative than the NOAEL process. This approach constructs mathematical models to fit all data points in the dose-response study and uses the most appropriate model to interpolate an estimate of the dose that corresponds to a particular level of response (a benchmark response), often 10%. A measure of uncertainty is also calculated, and the lower confidence limit on the benchmark dose is called the benchmark dose level (BMDL). The BMDL accounts for the uncertainty in the estimate of the dose-response that is due to characteristics of the experimental design, such as sample size. The BMDL can be used as the point of departure for derivation of a health-based guidance value or a margin of exposure (MOE)
Benzodiazepines	Group of GABA-agonist drugs.
BHA	Butylated hydroxyanisole, (1,1-dimethylethyl)-4-methoxyphenol) is an antioxidant used in many foods
BMD	Benchmark dose: an exposure due to a dose of a substance associated with a specified low incidence of risk, generally in the range of 1% to 10%, of a health effect, or the dose associated with a specified measure or change of a biological effect (IPCS, 2004).
BMDL	Benchmark dose level. Lower 95% confidence interval of the benchmark dose.
BMR	Benchmark response: the response, generally expressed as in excess of background, at which a benchmark dose or concentration is desired (IPCS, 2004).
BSEP	Bile salt export pump
CAR	Constitutive androstane receptor
Carcinogen	An agent that can cause malignant neoplasms (tumours). Examples of carcinogens include exogenous factors (chemicals, viruses, physical agents) and endogenous factors such as hormones.
Categorical regression	Statistical method for regression of ordered categories of toxic severity, to estimate the likelihood of a given category of severity at a given dose
CNS	Central nervous system
COC	The Committee on Carcinogenicity of Chemicals in Food, Consumer Products and the Environment. This is a committee of independent experts, which advises the UK Government on the carcinogenicity of chemicals

COM	The Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment. This is a committee of independent experts, which advises the UK Government on the mutagenicity of chemicals
COT	The Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment. This is a committee of independent experts, which advises the UK Government on the toxicity of chemicals
CSAF	Chemical-specific adjustment factor
CYP	Cytochrome P450
p'p'-DDE	The main and persistent metabolite of DDT
DDT	The ISO (International Organization for Standardization) name for the organochlorine insecticide dichlorodiphenyltrichloroethane
Debrisoquine	A drug used in the treatment of hypertension
DEET	The active ingredient in many insect repellent products. The chemical name is N,N-diethyl-meta-toluamide, and the ISO name is diethyltoluamide
Digoxin	A cardiac glycoside drug used to treat heart conditions, including cardiac failure. It increases the force of contraction of the heart muscle and reduces conductivity within the atrioventricular node.
DNA	Deoxyribonucleic acid: the carrier of genetic information for all living things (except the group of RNA viruses).
Dose	The total amount of an agent administered to, taken up or absorbed by an organism, system or (sub)population (IPCS, 2004).
Ecstasy	A drug of abuse: 3,4-methylenedioxymethamphetamine.
EFSA	European Food Safety Authority
EM	Endocrine modulator (also sometimes referred to as "endocrine disrupter"): a compound with the potential to alter hormone production, metabolism or action within the body
Epistemic uncertainty	In this report synonym for uncertainty. Literally, uncertainty pertaining to knowledge
ER	Estrogen receptor

EU	European Union
FAE	Fetal alcohol effects
FAO	Food and Agricultural Organization of the United Nations. The headquarters are in Rome
FAS	Fetal alcohol syndrome
fMRI	Functional Magnetic Resonance Imaging. This is a technique for determining which parts of the brain are activated by different types of physical sensation or activity
GABA	γ-aminobutyric acid, an inhibitory neurotransmitter
$GABA_A$	A type of GABA receptor
Genotoxic	The ability of an agent to damage the genetic material (eg. DNA or chromosomes) in an organism or cell, either directly or after metabolic activation.
GLP	Good Laboratory Practice
GSH	Glutathione
GST	Glutathione S-transferase, an enzyme
Hazard	The set of inherent properties of a substance or mixture of substances that makes it capable of causing adverse effects to humans, other organisms or the environment.
Hazard characterisation	The quantitative evaluation of adverse effects, by dose-response evaluation
Hazard identification	The identification of the type and nature of adverse effects that an agent has as inherent capacity to cause in an organism, system or (sub)population (IPCS, 2004).
HDUF	Human variability toxicodynamic adjustment factor
Health-based guidance values	Collective term for acceptable/tolerable daily intakes (ADI/TDI) and acute reference doses (ARfD). Also known as reference doses.
HIFα	Hypoxia inducible factor alpha. Hypoxia-inducible factor 1 is a transcription factor
Hyperekplexia	Startle disease, an inherited condition characterized by exaggerated startle response and due to abnormal glycinergic neurotransmission

Imposex	The occurrence of induced male sex characteristics superimposed on normal female gastropods, with the development of male sex organs, the penis and/or the vas deferens
INN	International non-proprietary name: agreed international name for drugs
In silico	Modelling research conducted using computers only
Intake assessment	Measurement or estimation of exposure to a chemical by any route for the population or subgroups thereof
IPCS	International Programme on Chemical Safety of the World Health Organization
IQ	Intelligence quotient
Isotretinoin	International non-proprietary name (INN) for a synthetic analogue of vitamin A, 13-cis-retinoic acid
JECFA	Joint FAO/WHO Expert Committee on Food Additives and Contaminants
JMPR	Joint FAO/WHO Expert Meeting on Pesticide Residues
LC-PUFA	Long-chain polyunsaturated fatty acid
d-Limonene	The major component of the oil extracted from citrus rind. d-Limonene is 1-methyl-4-isopropenyl-1-cyclohexene
3-MCPD	3-Monochloropropanediol, a contaminant of acid-hydrolysed vegetable protein
MDR1	Xenobiotic transporter P-glycoprotein (multidrug resistance protein 1)
Meta analysis	A quantitative method of combining the results of independent studies (usually drawn from the published literature) and synthesising summaries and conclusions. In the context of epidemiology, meta-analysis is a statistical analysis of the the results from independent studies, which aims to produce a single estimate of an effect.

MOE	Margin of exposure: The ratio between a defined point on the dose-response curve for the adverse effect, often the NOAEL or BMDL, and the estimated human intake. The MOE approach is a methodology that allows the comparison of the risks posed by different genotoxic and carcinogenic substances. It uses a reference point, often taken from an animal study and corresponding to a dose that causes a low but measurable response in animals This reference point is then compared with various dietary intake estimates in humans, taking into account differences in consumption patterns.
MOE_T	Total margin of exposure (the reciprocal of the sum of the reciprocals of the MOEs for the components of a mixture)
MRI	Magnetic resonance imaging
MRL	Maximum residue level for pesticides or maximum resilue limit for veterinary drug residues.
mRNA	Messenger RNA. The DNA of a gene is transcribed into mRNA molecules, which serve as the template for the synthesis of proteins.
MRP2	Multidrug resistance protein 2
Mutagen	An agent that can cause mutation.
Mutation	A permanent change to the amount or structure of the genetic material in an organism or cell, that can be passed on to the next generation of organisms or cells. The alterations may involve a single gene, a block of genes or whole chromosomes.
NAT	N-acetyltransferase, an enzyme
NOAEL	No observed adverse effect level: the highest concentration or amount of a substance, found by experiment or observation, that causes no detectable adverse alteration of morphology, functional capacity, growth, development or life-span of the target organisms under defined conditions or exposure (IPCS, 2004).
NRF2	Nuclear receptor factor 2
NTP	National Toxicology Program (USA)
OATP	Organic anion transporting polypeptide
OCT	Organic cation transporter

OECD	Organization for Economic Cooperation and Development. This organization developed from the Organisation for European Economic Co-operation (OEEC), which was formed to administer American and Canadian aid under the Marshall Plan. The headquarters are in Paris and amongst the activities of the OECD is production of internationally agreed protocols for toxicological tests
OP	Organophosphate (or organophosphorus). The term is usually used in pest control to describe derivatives of phosphoric and similar acids. The term covers a wide range of chemicals, including many with insecticidal and anticholinesterase activites.
OPIDP	OP-induced delayed polyneuropathy; A syndrome which occurs following exposure to certain OPs in which a central and peripheral motor and sensory neuropathy develops after a latent period
OPRM1	μ-Opioid receptor gene
Paracetamol	International non-proprietary name (INN) for an analgesic drug (Known as acetaminophen in the USA and some other parts of the world)
Paraoxon	The oxon (active metabolite) of an OP insecticide, parathion
Paraoxonase	Esterase with the capability of hydrolysing organopshophates *inter alia* paraoxon
Parkinson's disease	Neurological disease characterised clinically by rigidity and tremor and pathologically by loss of dopaminergic neurones in the substantia nigra of the brain.
PBPK	Physiologically-based pharmacokinetics
PBTK	Physiologically-based toxicokinetics
PCB	Polychlorinated biphenyl
PCR	Polymerase chain reaction. A method for creating millions of copies of a particular segment of DNA. PCR can be used to amplify the amount of a particular DNA sequence until there are enough copies available to be detected.
PCR-RFLP	PCR-Restriction fragment length polymorphism. Also known as cleaved amplified polymorphic sequencing (CAPS).
Phase I metabolism	Biotransformation reactions in which a polar, reactive group is introduced into a molecule or a molecule is broken into smaller molecules. The resulting metabolite is usually more soluble and may take part in phase II metabolism.

Phase II metabolism	Conjugation reactions that attach a chemical group (eg. glucuronide, sulphate, an amino acid, glutathione) to a molecule. The resulting conjugated metabolite is usually more water soluble and more readily excreted.
Phenobarbital	International non-proprietary name (INN) for an anticonvulsant drug
Phenytoin	International non-proprietary name (INN) for an anticonvulsant drug
pKa	The pKa of a substance is the pH at which half of the molecules are in one ionised form, the other half being unionised or in a different ionised form
Polymorphism	A genetic variant where the less common allele appears in at least 1% of a population.
PON1	Paraoxonase 1
PPARα	Peroxisomal proliferator activated receptor alpha
PXR	Pregnane X receptor
QQ	A paraoxonase 1 genotype
QR	A paraoxonase 1 genotype
Reference dose	Collective term for acceptable daily intakes (ADI), tolerable daily intakes (TDI) and acute reference doses (ARfD). Also known as health-based guidance values.
Risk	Possibility that a harmful event (death, injury or loss) arising from exposure to a chemical or physical agent may occur under specific conditions.
Risk characterisation	The qualitative and, wherever possible, the quantitative determination, including attendant uncertainties, of the probability of occurance of known and potential adverse effects of an agent in a given organism, system or (sub)population, under defined exposure conditions (IPCS, 2004). The consideration of hazard identification, hazard characterisation and intake assessment in combination to predict whether effects in humans are likely, the severity and nature of such effects, the proportion of the population affected and the existence of any vulnerable sub-populations
RNA	Ribonucleic acid: a molecule similar to DNA, which helps in the process of decoding the genetic information carried by DNA.
RR	A paraoxonase 1 genotype
RXR	Retinoic acid X receptor

St John's Wort	A herbal remedy for depression obtained from *Hypericum perforatum* (St. John's wort), a plant native to Europe
SARs	Structure-activity relationships
SCF	The European Commission's Scientific Committee on Food (formerly the Scientific Committee for Food)
SHBG	Sex hormone-binding globulin.
SNP	Single nucleotide polymorphism
Sponsor	In regulatory toxicology, a generic term for organisations responsible for providing the data on regulated chemicals. The sponsor is usually a public company, less often a group of companies.
Sumatriptan	INN name for a drug used in the treatment of migraine: the drug is a 5-hydroxytryptamine1 receptor subtype agonist.
TBT	Tributyl tin, a fungicide
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TDI	Tolerable daily intake: Estimated maximum amount of a food contaminant, often expressed on a body mass basis, to which an individual may be exposed daily over a lifetime without appreciable risk to health
TDS	Testicular dysgenesis syndrome: A syndrome comprising various reproductive disorders including low sperm counts and testicular germ cell cancer in young adult men and incomplete testicular descent (cryptorchidism) and abnormal opening of the urethral meatus on the penis (hypospadias)
Toxicodynamics	The process of interaction of chemical substances with target sites and the subsequent reactions leading to adverse effects (IPCS, 2004).
Toxicokinetics	The description of the fate of chemicals in the body, including a mathematical account of their absorption, distribution, metabolism and excretion.
UDPGT	UDP-glucuronysyltransferase, an enzyme
Uncertainty	Imperfect knowledge concerning the present or future state of an organism, system or (sub)population under consideration (IPCS, 2004).

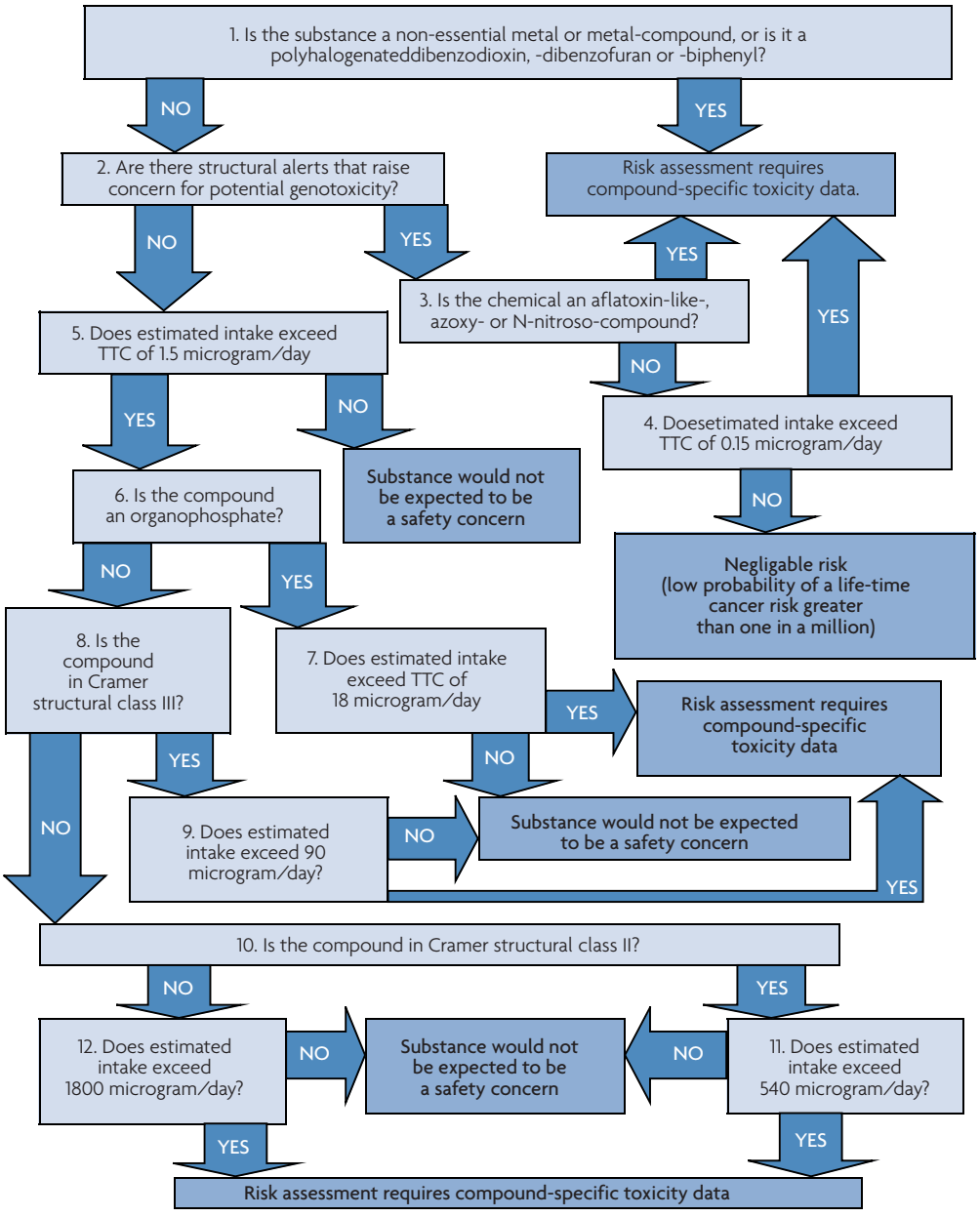
Uncertainty factor	Value used in extrapolation from experimental animals to man (assuming that man is more sensitive) or from selected individuals to the general population: for example, a value applied to the NOAEL to derive an ADI or TDI. The value depends on the size and type of population to be protected and the quality of the toxicological information available.
USEPA	The United States Environmental Protection Agency
Variability	Observable diversity in biological sensitivity or response, and in exposure parameters (IPCS, 2004).
VDR	Vitamin D receptor
VICH	International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products. The VICH harmonizes requirements for toxicological and other tests in relation to veterinary drugs.
Vinclozolin	The ISO (International Organization for Standardization) name of a fungicide
VUT	The Working Group on Varability and Uncertainty of the Committee on Toxicity of Chemicals in Food, Consumer products and the Environment
WHO	World Health Organization
XME	Xenobiotic metabolising enzymes

Reference

IPCS, 2004, "Principles for Modelling Dose-Response for the Risk Assessment of Chemicals", draft Environmental Health Criteria document of the International Programme on Chemical Safety, World Health Organisation, Geneva, February 2004.

Appendix 6

Decision tree for applying the threshold of toxicological concern (TTC)



"Decision tree for low molecular weight compounds for which limited toxicity data are available, that incorporates different thresholds of toxicological concern related to different structural characteristics".

Reprinted from Food and Chemical Toxicology, volume 42 (issue 1), Kroes R, Renwick AG, Cheeseman M, Kleiner J, Mangelsdorf I, Piersma A, Schilter B, Schlatter J, van Schothorst F, Vos JG and Wurtzen G, "Structure-based thresholds of toxicological concern (TTC): guidance for application to substances present at low levels in the diet", page 76, Copyright 2004, with permission from Elsevier.

Appendix 7

Membership of COT working group on variability and uncertainty in toxicology.

CHAIRMAN

Professor P Aggett OBE MSc FRCP FRCPCH
Head of Lancashire School of Health and Postgraduate Medicine, University of Central Lancashire (past Vice-Chairman of COT)

MEMBERS

Professor A Boobis OBE BSc PhD CBiol FIBiol
Professor of Biochemical Pharmacology, Imperial College, London. (COT member)

Professor M Hadders-Algra MD PhD
Professor of Developmental Neurology, University Medical Centre, Groningen, The Netherlands.

Dr P Jackson BA(Oxon) MA(Oxon) MB ChB MRCP PhD FRCP FFPM(Dis)
Reader in Clinical Pharmacology and Theraputics, Royal Hallamshire Hospital, University of Sheffield. (COT member)

Professor D Ray BSc PhD
Head of Applied Neuroscience Group, University of Nottingham Medical School. (COT member)

Professor A G Renwick OBE BSc PhD DSc
Emeritus Professor, School of Medicine, University of Southampton. (Past COT Member)

Professor M Rowland BPharm MSc PhD DSc FRPharmS FIMA
Centre of Applied Pharmacokinetic Research, University of Manchester

Dr L Rushton OBE BA MSc PhD CStat
Principal Research Fellow, Department of Public Health and Epidemiology, Imperial College London. (COT member)

Ms J Salfield BSc MSc MIFST CertED RPCPCH (until 31 March 2004)
Public Interest Representative. (past COT member)

Dr R Sharpe BSc MSc PhD
Medical Research Council Human Reproductive Science Unit, University of Edinburgh.

Dr L Stanley BA PhD
Consultant in Investigative Toxicology. (COT member)

Ms A Ward BA
Public Interest Representative. (COT member)

ASSESSORS

Mr J Battershill BSc MSc
Department of Health

Dr S Brescia
Health and Safety Executive

Mr A Browning IDT CBiol MIBiol
DEFRA – Veterinary Medicines Directorate

Dr R Brown
DEFRA – Pesticide Safety Directorate

SECRETARIAT

Professor TC Marrs OBE DSc MD FRCP FRCPath FIBiol (until 31 August 2005)
Mr DW Renshaw BSc CBiol MIBiol (from 1 September 2005)
Dr D Benford BSc PhD (Scientific Secretary of COT).
Members of the secretariat were all from the Food Standards Agency, London.

Appendix 8

Declaration of COT working group on variability and uncertainty in toxicology

	PERSONAL INTERESTS		NON PERSONAL INTERESTS	
Member	**Company**	**Interest**	**Company**	**Interest**
Professor P Aggett (Chair)	NONE	NONE	AstraZeneca Smith Nephew Nestec ILSI Abbott Wellcome Yakult International Copper Association	Lecture fees Lecture fees Lecture fees Lecture fees Lecture fees Lecture fees Lecture fees Lecture fees
Professor A Boobis OBE	Bank Santander Scottish Power Centrica BG Group Halifax Barclays National Grid Transco BT Group Astellas Pharma	Shares Shares Shares Shares Shares Shares Shares Shares Shares Shares	GlaxoSmithKline FSA ILSI HESI Elsevier	Support by Industry Research Contract Member of Board of Trustees Editor-in-Chief; Food and Chemical Toxicology
Professor M Hadders-Algra	NONE	NONE	NONE	NONE
Dr P Jackson	British Heart Foundation Mitchell & Butler Intercontinental Hotels Marks & Spencer	Lecture Fees Share Ownership Share Ownership Share Ownership	Medtronic AVE	Research Grant
Professor D Ray	Medical Research Council	Employer	Consortium of: Bayer, DuPont, FMC Syngenta & Valent	Research Grant
Professor A G Renwick	International Sweetners Association (ISA) Redbull Ajinomoto Unilever Nestlé Coca-Cola	Consultant Consultant Consultant Consultant Consultant Member of Scientific Advisory Council	NONE	NONE

	PERSONAL INTERESTS		NON PERSONAL INTERESTS	
Member	**Company**	**Interest**	**Company**	**Interest**
Professor M Rowland	Alpro Foundation	Consultancy	ILSI Europe	Partner in EC funded project
	Woolwich	Shares	Cerestar (Belgium)	Funded Research
	Halifax	Consultancy	Geest	Funded Research
	Unilever	Consultancy	Vitacress	Funded Research
	Glanbia	Consultancy	Yakult UK	Funded Research
	Clasado	Consultancy	Nicobrand	CAST PhD studentship
			Scottish Crops Research	CAST PhD studentship
Dr L Rushton	Northern Rock	Shares	European Silica	Ongoing Cohort Study
	Friends Provident	Shares	International Manganese Institute	Contract to IEH to prepare criteria document – completed
	Unilever	Consultancy – advice on design of an epidemiological survey relating to dermatitis	American Chemistry Council	Contract to IEH for systematic review and meta analysis – completed
Ms J Salfield	NONE	NONE	NONE	NONE
Dr R Sharpe	National Institute for Environmental Health Science (USA) via Constella Group	Ad-Hoc Consultancy	NONE	NONE
	Encysive Pharmaceuticals (USA)	Ad-Hoc Consultancy		
	Takeda Global Research & Development (Chicago & London)	Ad-Hoc Consultancy		
	Standard Life	Shares		
Dr L Stanley	CXR Biosciences	Salary	Alizyme	Company Contract
	Agan	Company Contract	Arrow	Company Contract
	Procter & Gamble	Company Contract	Bayer	Company Contract
	Toxel	Company Contract	Cyclacel	Company Contract
	Association of Plastics Manufacturers, Europe Eurochlor	Consortium Client	Entremed	Company Contract
	European Council for Plasticisers and Intermediates	Consortium Client	Etiologics	Company Contract
	Halogenated Solvents Industry Association	Consortium Client	Ferring	Company Contract
	AstraZeneca	Research Collaboration	Grupovita	Company Contract
	GlaxoSmithKline	Research Collaboration	Guerbet	Company Contract
	NovoNordisk	Research Collaboration	Ionix	Company Contract
	Wyeth	Research Collaboration	Nestle	Company Contract
	Pfizer	Research Collaboration	Neuroseach	Company Contract
			Oncosense	Company Contract
			Serono	Company Contract
			Stiefel	Company Contract
			Strakan	Company Contract
			Yamanouchi	Company Contract
Ms A Ward	NONE	NONE	Barking, Havering and Redbridge NHS Trusts	Non-Executive Director

Appendix 9

Membership of Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment (COT)

CHAIRMAN

Professor IA Hughes MA MD FRCP FRCP(C) FRCPH F Med Sci
Professor and Head of Department of Paediatrics, University of Cambridge.

MEMBERS

Professor J Ashby BSc PhD CChem FRCS (until February 2006)
Research Fellow, Syngenta.

Dr D Bell BSc PhD
Reader in Molecular Toxicology, University of Nottingham.

Professor A Boobis OBE BSc PhD CBiol FIBiol
Professor of Biochemical Pharmacology, Imperial College, London

Dr P Carthew BSc MSc PhD FRCPath
Senior pathologist, SEAC Toxicology Unit, Unilever

Dr Dearman BSc PhD
Head of Immunology, Syngenta.

Dr J Hinson BSc PhD DSc
Reader in Molecular and Cellular Endocrinology, Barts and the London, Queen Mary School of Medicine and Dentistry, University of London

Dr J Foster BSc PhD FRCPath (from February 2006)
Senior Principal Pathologist, AstraZeneca

Dr P Jackson BA(Oxon) MA(Oxon) MB ChB MRCP PhD FRCP FFPM(Dis)
Reader in Clinical Pharmacology and Theraputics, Royal Hallamshire Hospital, University of Sheffield.

Professor J Lunec BSc PhD FRCPath
Head of Molecular Toxicology, King's College, London.

Professor D Ray BSc PhD
Head of Applied Neuroscience Group, University of Nottingham Medical School.

Professor M Rowland Bpharm MSc PhD DSc FRPharmS FIMA
Centre of Applied Pharmacokinetic Research, University of Manchester

Dr L Rushton OBE BA MSc PhD CStat
Principal Research Fellow, Department of Public Health and Epidemiology, Imperial College, London

Dr G Rylance MBChB MRCP FRCOCH (until February 2006)
Head of Epidemiology, Medical Research Council, Institute for Environment and Health, University of Leicester

Ms J Salfield BSc MSc MIFST CertED RPCPCH (until 31 March 2004)
Public Interest Representative.

Dr L Stanley BA PhD
Consultant in Investigative Toxicology

Professor S Strobel MD PhD FRCP FRCPCH
Institute of Child Health, London

Dr C de Vries MSc PhD
Senior lecturer in pharmacoepidemiology, University of Surrey

Ms A Ward BA
Public Interest Representative

Ms A Williams OBE BA
Public Interest Representative

SECRETARIAT

Dr D Benford BSc PhD – FSA Scientific Secretary
Food Standards Agency (FSA)

Mr J Battershill BSc MSc – DH Scientific Secretary
Department of Health (DH) – until 2006
Health Protection Agency (HPA) – from 2006

Mr KV Butler – administrative secretary – until March 2006
Food Standards Agency (FSA)

Mrs J Shroff – administrative secretary – from March 2006
Food Standards Agency (FSA)

Appendix 10

List of those who provided substantive comments on the draft report as part of the consultation exercise

Date received	Name of individual/organisation
20 April 2006	Dr Douglas McGregor, consultant toxicologist.
2 May 2006	Professor David Coggan, MRC Epidemiology Resource Centre, Southampton General Hospital.
6 June 2006	Dr Michael Festing, MRC Toxicology Unit, University of Leicester.
23 June 2006	Miss Margaret J Reichlin, member of the public.
6 July 2006	Mr Richard AR Bruce, member of the public.
7 July 2006	Dr Anna Rowbotham & Dr George Loizou, Health and Safety Laboratory, Buxton.
9 July 2006	Dr Geoff H Pigott, consultant toxicologist.
10 July 2006	Nirmala Bhogal & Robert Coombes, Fund for the Replacement of Animals in Medical Experiments (FRAME), Nottingham.
10 July 2006	Professor Jon Ayres, Chairman of the Advisory Committee on Pesticides (ACP).
15 July 2006	Margaret Anderson, member of the public.
11 August 2006	Georgina Downs, UK Pesticide Campaign

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http://www.food.gov.uk/science/ouradvisors/toxicity/COTwg/wgvut/

If you require any further information about the work of the committees, or the contents of this report, please write to the committee's administrative secretary at the following address:

COT Secretariat
Food Standards Agency
Room 511C
Aviation House
Kingsway
London WC2B 6NH
Tel: 0207 276 8522
Fax: 0207 276 8513
e: Julie.Shroff@foodstandards.gsi.gov.uk

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