Toxicological Modes of Action: Relevance for Human Risk Assessment

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Toxicological Modes of Action: Relevance for Human Risk Assessment

CONTENTS

EXECUTIVE SUMMARY 1

1. INTRODUCTION 3

1.1 Background 3
1.2 Scope of report 4

2. DIFFERENCES IN SENSITIVITY BETWEEN ANIMALS AND HUMANS 6

2.1 Toxicokinetic differences 6
2.2 Toxicodynamic differences 6
2.3 Implications of toxicokinetic and toxicodynamic species differences 7

3. EXTRAPOLATION OF A TOXIC EFFECT OBSERVED IN ANIMALS TO HUMANS 8

3.1 Identification of mode of action 8
3.2 Postulation and testing of a mode of action hypothesis 9
  3.2.1 Proposed mode of action 11
  3.2.2 Dose-response relationship 11
  3.2.3 Temporal association 11
  3.2.4 Strength, consistency and biological plausibility 11
  3.2.5 Eliminating other modes of action 12
  3.2.6 Outcome of mode of action assessment 12
3.3 Assessment of human relevance 12

4. THE APPROACH IN PRACTICE 15

4.1 An established mode of action 15
  4.1.1 $\alpha_2u$-Globulin-mediated nephropathy in male rats 17
  4.1.2 Forestomach tumours 18
  4.1.3 Thyroid gland stimulation by enzyme inducers 20
  4.1.4 Tyrosinaemia and corneal opacity in the rat 22
  4.1.5 Other modes of action where relevance for humans has been questioned 23
4.2 An observed effect with an unknown mode of action 26

5. CONCLUSION 27

ABBREVIATIONS 29
APPENDIX A: TOXICOKINETIC DIFFERENCES BETWEEN ANIMALS AND HUMANS

Coumarin 30
Methylene chloride 31
Formaldehyde 32
Phenoxyacetic acids 32

APPENDIX B: TOXICODYNAMIC DIFFERENCES BETWEEN ANIMALS AND HUMANS

α2u-Globulin-mediated kidney tumours in male rats 34
Forestomach tumours 34
Thyroid gland stimulation by enzyme inducers 35
Triketone-mediated tyrosinaemia and corneal opacity 36

BIBLIOGRAPHY 38

MEMBERS OF THE TASK FORCE 51

MEMBERS OF THE SCIENTIFIC COMMITTEE 52
EXECUTIVE SUMMARY

Generally, the assessment of hazard and risk to humans that might arise from exposure to chemical substances is based on the extrapolation from data generated in studies with experimental animals. The assumption is made that the effects observed in the animals would also be expressed in humans and, to account for uncertainties including intra- and interspecies variability, and more recently susceptible human subpopulations, assessment factors are applied in deriving ‘safe’ exposures for humans.

This is usually done without testing the hypothesis in humans, as the default approach allows for humans to be more sensitive than the most susceptible animal species. However, for those substances where humans are not sensitive to the effects seen in the animal studies, this approach is conservative and may result in risk management measures being taken that are totally unwarranted. An alternative approach is proposed in this report for the evaluation of such substances.

The view has been expressed by many leading researchers and regulatory organisations that an understanding of the molecular and cellular processes underlying toxicity and carcinogenicity allows a more scientifically-based risk assessment and, where such information is available and adequate, this should replace default approaches in assessing human risk.

All mechanisms of toxicity can be described in terms of a sequence of events, each of which is critical to the manifestation of the toxic endpoint. To elucidate such a mechanism requires rigorous investigation to obtain a complete and detailed understanding of the process leading to a toxic effect. However, the complete elucidation of all the events leading to the effect is not essential for evaluating the species differences in sensitivity to a toxicant. To achieve this we can restrict our understanding to selected key events within the complete sequence of events. This concept, termed the ‘mode of action’ can be used for the evaluation of species specificity including human susceptibility. If it could be shown that one or more of the key events could not occur in humans, or could only occur to a much lesser extent, it could be assumed that the mode of action of the substance in question was not relevant to the assessment of human risk. In such cases departure from the default approach would be justified.

This philosophy is illustrated by a number of well-studied examples for which the modes of action and corresponding key events have been established for effects that are not relevant to humans.

A structured two-step ‘mode of action’ approach was developed to guide the evaluation of substances expressing toxicity suspected as being species-specific and of questionable relevance to humans. Advice is given on how to identify the key events in the mode of action of the
toxicological effect, how to test the strength of the data on which the mode of action hypothesis is based and how to establish its relevance to humans.

The extent to which new data will be required to associate a substance with an established mechanism of toxicity or mode of action will vary, depending on the existing knowledge of the role of toxicokinetics and toxicodynamics and of the understanding already available within that class of chemicals.

The use of this approach to identify those substances which express toxicity in experimental animals via a mode of action that is not relevant to humans will enable a more scientifically objective assessment of risk in humans. Furthermore, adopting a ‘mode of action’ approach should considerably speed up the risk assessment process and avoid unnecessary animal experimentation.
1. INTRODUCTION

1.1 Background

Fundamental to the evaluation of the safety of chemicals is an understanding of their intrinsic toxicological properties. Commonly, such data are derived from toxicological studies carried out to comply with requirements of national and international regulatory organisations such as the US EPA, JMAFF and EC. In general observations are made in laboratory animals exposed to a range of dosages or concentrations by the most appropriate route, or routes, of administration taking into account likely human exposure patterns. The inclusion of more than one species can provide reassurance that a critical effect for humans will not be missed by studying only a potentially insensitive species. The data generated in such studies are used in two ways. Firstly, to determine the potential hazard of the substance by taking into account the route of exposure and nature of any observed adverse effects; secondly, to gain an understanding of the potential risks to man by comparing the dose at which these effects occur with known or estimated human exposures. The critical determining factors in the second step will be the adverse effect(s) for which the protection of the exposed population is required and the exposure level(s) at which such effects do and do not occur.

In the absence of comparative data in humans, simple and conservative ‘worst case’ assumptions are made, the most fundamental being that all adverse effects of a substance that are observed in animal studies, are relevant for humans. Where more than one adverse effect arises from exposure to the substance under evaluation, the assessment of hazard and risk to humans is generally based on the ‘lead’ effect (toxic effect of concern), i.e. the most serious effect evoked at the lowest exposure level. Where data from more than one species or strain are available, the assessment is based on the most susceptible of these.

In assessing the risk to humans, this conservative procedure is reinforced through the application of ‘safety’, ‘assessment’ or ‘uncertainty’ factors to take account of uncertainty over interspecies and intraspecies differences in sensitivity to the test substance (WHO, 1987; Renwick and Lazarus, 1998). Further details and guidance on the derivation and application of appropriate assessment factors in human health risk assessment can be found in ECETOC Technical Report No 86 (ECETOC, 2003).

For many substances the default approach provides adequate margins of safety for the potentially exposed population for the defined use of the chemical. However, in some situations, e.g. where humans are demonstrably much less sensitive than the test species or, indeed, where it is known that the effects seen in the test animal would under no circumstances be manifest in humans, such conservatism could lead to risk management measures that are unnecessary.
There are a growing number of chemicals where application of the usual default approach to risk assessment has been shown to be too conservative; many of these cases have been recognised formally by regulators and by international organisations.

Scientists from CIIT were among the first to publish a strategy in which risk assessment approaches for animal carcinogens were founded on an understanding of their modes of action (Butterworth et al., 1995). In 1996 and 1999, US EPA included the use of mechanistic data in their ‘Proposed Guideline for Carcinogenic Risk Assessment’ (US EPA, 1996, 1999). Meanwhile the application of mechanistic / mode of action data was further recommended and extended to the assessment of non-cancer endpoints (Schlosser and Bogdanffy, 1999; Haber et al., 2001; Sonich-Mullin et al., 2001).

A common theme of these proposals is that an understanding of the molecular and cellular processes underlying toxicity and carcinogenicity allows a more scientifically-based risk assessment and, where such information is available and adequate, this should replace default approaches in assessing human risk.

Against this background a Task Force was commissioned by ECETOC to examine, in the context of human risk assessment, the role and use of mode of action and mechanistic data in establishing the relevance of toxicological effects observed in experimental animals.

The Task Force was assigned the following Terms of Reference:

- Illustrate, with examples, how it can be demonstrated that a mode of action observed in an animal model is not relevant, or cannot be extrapolated to humans.
- Provide a rational scientific approach by which it might be judged to what extent a mode of action seen in an animal model can be considered not relevant for humans.

1.2 Scope of report

Conventionally, justification for excluding positive toxicity data on the grounds of non-relevance to humans requires extensive mechanistic data that provide a complete and detailed understanding of all the steps in the sequence of events leading to the toxic effect. There are relatively few substances for which such comprehensive toxicological data exist. However, the complete elucidation of all the events preceding toxicity is not believed to be essential to establish major differences in sensitivity between species.
Two main aspects of toxicity, toxicokinetics and toxicodynamics, can account for the nature and extent of differences between species in their sensitivity to substances. The implications for risk assessment are considered in the opening chapters of the report.

The expression of toxicity in a mammalian system is dependent on a sequence of key events taking place, each of which is critical to the manifestation of the toxic endpoint. Thus, for a specific toxic endpoint, to justify deviating from the default approach in risk assessment, it should be sufficient to identify the key events in the process (mode of action) and establish that these would not occur in humans, or that they would occur only to a much lesser extent. From such knowledge it could be concluded that the toxic outcome would not be observed in humans, or be observed only at much higher and possibly irrelevant exposure levels. A proposal is made, illustrated with well studied examples, for a structured approach for establishing and reviewing the mode of action by which a substance causes a toxicological effect. This allows a decision to be made on the appropriate animal endpoint for human hazard identification and risk assessment. This concept is elaborated in the context of extrapolation of toxicity data from animals to humans, and guidance given on testing the hypothesis on which the non-relevance to humans is based.

Throughout this report, a clear distinction is made between two terms often used in this context, i.e. ‘mechanism of toxicity’ and ‘mode of action’ of a substance. Identifying a ‘mechanism of toxicity’ requires rigorous investigation to obtain a comprehensive understanding of the entire sequence of events that result in the toxic effect of interest. In practice, ‘mechanisms of toxicity’ are rarely established. For the ‘mode of action’ concept it is sufficient to develop an understanding of key events within the complete sequence of events leading to toxicity. This does not require the amount or depth of data needed to provide a detailed explanation of the complete sequence of events.

This document is intended as a reference for industrial, academic and regulatory toxicologists, as well as those individuals less familiar with the evaluation process, to aid the formulation and application of consistent judgements in the interpretation of toxicity data and their extrapolation to humans. This initiative is one of three component parts of a project aimed at providing guidance on the process of human risk assessment of chemical substances. The two other parts looked at adverse vs. non-adverse effects in toxicological studies, published as Technical Report No. 85 (ECETOC 2002) and risk assessment factors for human health risk assessment, published as Technical Report No. 86 (ECETOC 2003).
2. DIFFERENCES IN SENSITIVITY BETWEEN ANIMALS AND HUMANS

The expression of toxicity arising from exposure to a chemical substance is a consequence of a chain of events that results in the affected tissues of an organism receiving a sufficient quantity of that substance (or its ultimate toxic metabolites) to produce the adverse effect. The factors that confer species susceptibility, and lead to major differences between animals and humans in their response to such chemical insults, are those that modulate these critical requirements, either in the nature and quantity of the ultimate toxicant that is presented to the sensitive tissues (toxicokinetics) or in the sensitivity of those tissues to the ultimate toxicant, i.e. the toxicodynamic response.

2.1 Toxicokinetic differences

The toxicokinetic phase begins with exposure and results in a certain concentration of the ultimate toxicant at the target site. This concentration is dependent on the absorption, distribution, metabolism and excretion (ADME) of the substance. Species differences are known to occur at each stage within this sequence, leading to different target site concentrations of the parent compound or its critical metabolites.

There are several well-investigated substances where toxicity is determined by different species-specific toxicokinetic profiles. In the case of a major quantitative or even a qualitative species difference, a toxic metabolite might be present only at low concentrations or even below the limit of detection in the non-responsive species. In other cases, moderate or minor quantitative differences explain quantitative differences in toxic response.

Coumarin and methylene chloride are examples of substances where tumour formation appears to be the consequence of metabolic activation that is specific to rodents. The particular susceptibility of dogs to adverse effects of phenoxyacetic acids and related compounds results from a markedly lesser ability of this species (compared to others) to excrete organic acids. These examples are well documented in the literature and are summarised in Appendix A.

2.2 Toxicodynamic differences

In the toxicokinetic phase (Section 2.1) the bioavailability of the substance or its toxic metabolite(s) characterises the species difference in response. The toxicodynamic phase comprises all events, subsequent to the delivery of the ultimate toxicant to the target tissues, which finally lead to the toxicological effect; these include interaction of the substance / toxic metabolite(s) with the target tissues (comprising cells, organelles and biological macromolecules...
such as enzymes, receptors and DNA) and all patho-physiological consequences thereof, ultimately leading to the expression of toxicity. A species difference in response can arise from a difference in the interaction of a substance with the target, e.g. where the respective molecular target is absent in the non-susceptible species. Even if the target is present and the substance does interact, the biochemical and/or patho-physiological consequences may be different in different species.

There are several well-investigated examples where species-specific toxicity is explained either by qualitative or by major quantitative differences at the target site. Two such examples are α2u-globulin nephropathy in the male rat, and the development of tumours of the forestomach in rodents; the target protein and the target tissue, respectively, are not present in humans. In other examples, such as the formation of thyroid follicular cell tumours by liver enzyme inducers in the rat or triketone-mediated tyrosinaemia leading to corneal opacity in the rat, the arguments for non-relevance for humans rely on cellular and/or molecular aspects, as well as on knowledge from human aetiology and epidemiology (see Section 4, and Appendix B for further details of these and other examples).

2.3 Implications of toxicokinetic and toxicodynamic species differences

In the examples quoted in Section 4 and Appendices A and B, the weight of evidence demonstrates clearly that a chemically induced toxic effect in an animal species is unlikely to occur in humans. This lower or even absence of susceptibility in humans is the result of a fundamental difference between animals and humans in either ADME or in the toxicodynamic effect of the substance and its biotransformation products.

Where the species difference is due to differences in bioavailability (e.g. metabolic activation of methylene chloride uniquely in mice), the arguments in favour of non-relevance for humans are substance-specific. However, where there is a difference in toxicodynamics and the established mode of action is unique for the species and sex (e.g. chemically induced α2u-globulin nephropathy and kidney tumours in the male rat), arguments in favour of non-relevance to humans are species- / sex-specific. Thus, where any substance is shown to be nephrotoxic and causes kidney tumours only in the male rat, it can be concluded that such a finding is not relevant in the assessment of the human risk of that substance, provided that it can be shown that the mode of action is via the formation of α2u-globulin.

In some cases, the establishment of non-relevance to humans is possible only with quantitative analysis of extensive data on a range of parameters. One such example is formaldehyde where target tissue dosimetry (quantification of DNA-protein cross-links) together with species differences in physiological parameters, were analysed using physiologically based kinetic modelling; this enabled the derivation of more appropriate risk estimates for humans in place of application of the default assessment factors.
3. EXTRAPOLATION OF A TOXIC EFFECT OBSERVED IN ANIMALS TO HUMANS

The potential risk to human health from exposure to chemical substances is generally assessed by extrapolation from animal data to humans. Two approaches can be used for such extrapolation. In the first, which is the default approach, the assumption is simply made that all effects observed in animals are relevant to humans. To account for interspecies and intraspecies differences in sensitivity to the substance, numerical factors are used to establish safe levels of exposure for humans. The derivation of such generic assessment factors and their application in human health risk assessment is the subject of Technical Report No. 86 (ECETOC, 2003) and is not discussed further in this report.

If the available data suggest that the effect observed is of questionable relevance to humans, a second approach may be appropriate in which the mode of action underlying the effect is examined and taken into account to justify departure from the first (default) approach. In the following sections this second approach is presented in detail.

3.1 Identification of mode of action

The first stage in the process of extrapolating a toxic effect observed in animals to humans is the identification of the mode of action that results in the adverse effect(s) observed in the susceptible species. If the mode of action is unknown, the test organism can be considered as a ‘black box’, i.e. a system that, on exposure to a sufficiently high amount of the substance, responds with the effect (Figure 3-1).

Figure 3-1: Black box (1)

A sequence of key molecular and cellular events takes place within the black box, beginning with exposure to the substance and ending with the expression of toxicity (Figure 3-2). Each key event in the process can be assigned either to the toxicokinetic or to the toxicodynamic phase.
The concept of describing the entire process from exposure to toxicological effect as a sequence of key events implies that the effect will be observed only under conditions where each of these key events within the sequence proceeds to a sufficient extent. In other words, if within this sequence of key events, one critical event does not take place, or does not take place to a sufficient extent, the ultimate toxicological effect will not be observed (Figure 3-3).

The phenomenon of species differences in toxicity can be examined in the context of this process. Within this concept, any species difference must be the consequence of a difference in the response to at least one of the key events.

### 3.2 Postulation and testing of a mode of action hypothesis

The adoption, or rejection, of a proposed mode of action is based on the toxicity data available for the substance. The major part of such data generally consists of studies performed according to recognised regulatory guidelines. In addition, relevant substance-specific information may be available from toxicokinetic and metabolic studies and from specially designed in vivo and/or in vitro mechanistic or mode of action studies. This can be supplemented by data from structurally-related substances and from substances with similar toxicological profiles. Human data for the substance or related substances are highly relevant and where available, should be included in the assessment.
The conclusion as to the strength of the data in support of the postulated mode of action (Scheme 3-1) is reached essentially by following the ‘Hill Criteria’ (Hill, 1965), adapted for mode of action analysis in experimental toxicology (Schlosser and Bogdanffy, 1999) and cancer risk assessment (Sonich-Mullin et al, 2001. These principles are described in the following subsections.

**Scheme 3-1**

1. Identify lead effect of concern
2. Postulate mode of action (hypothesis)
3. Test mode of action hypothesis
   - Dose-response relationship
   - Temporal association
   - Strength and consistency, biological plausibility
   - Other modes of action
4. Accept or reject postulated mode of action
3.2.1 Proposed mode of action

The key events in the proposed mode of action should be defined clearly and any species differences highlighted in the expression of the toxic effect of concern. Such key events should be quantifiable.

3.2.2 Dose-response relationship

In relation to both the parent substance and the ultimate toxicant, a comparison of dose-response information in susceptible animals is critical to support or invalidate the proposed mode of action. For example, for a substance that requires metabolic activation, the variation in the production or removal of the ultimate toxicant is critical to the difference in the toxicological response of the respective species.

If a key event is postulated to be critical to the mode of action, it should be observed at all dose levels, including the lowest, at which the ultimate toxic effect is expressed.

3.2.3 Temporal association

The key events that are essential for the expression of toxicity must occur in a plausible sequence that is consistent with the proposed mode of action.

Postulated key events should be observed after shorter treatment periods than those that cause the ultimate toxic effect.

3.2.4 Strength, consistency and biological plausibility

The more extensively a hypothesis is challenged experimentally, the greater will be the confidence in the postulated mode of action. Both direct and indirect evidence can be of value in ascertaining the strength and consistency of the proposed mode of action.

Modulation of the toxicological effect can be a useful experimental challenge of a mode of action hypothesis. For example, the blocking of a key event, such as metabolic activation, should result in significant diminution of the toxicological effect. In contrast, induction of the metabolic step should increase the extent of bioactivation and hence the toxicological response.

The proposed mode of action should not conflict with generally accepted facts or principles relating to the pathogenesis of the ultimate toxicological effect and should be internally consistent.
with other aspects of the toxicology dataset for that substance. If the mode of action is relevant to a family of structurally-related substances, then data on the toxic effects of other family members would be expected to complement and strengthen the existing data on the substance in question.

### 3.2.5 Eliminating other modes of action

The strengths, plausibility and coherence of alternative modes of action that might result in the same toxicological effect should be examined. For example, if tumour formation was the toxic endpoint and a non-genotoxic mode of action was postulated, data should be available that support the exclusion of genotoxicity as the mode of action.

### 3.2.6 Outcome of action assessment

The outcome of the assessment might be that the existing data provide a convincing or reasonable argument in favour of the postulated mode of action.

In cases where the data leave important questions unanswered, or where the data raise some inconsistencies, additional information will be needed before the postulated mode of action can be accepted.

### 3.3 Assessment of human relevance

Once a mode of action has been established for the lead effect in experimental animals, its relevance to humans should be assessed. The human relevance of the effect can be challenged if at least one identified key event does not take place, or does not take place to a sufficient extent, in humans. Such a key event might be identified at any point in the sequence of events that takes place between exposure to the substance and expression of the toxic effect.

To demonstrate the existence of such a key event is challenging, particularly for substances where the results of testing in humans are not available or appropriate. A straightforward example is where a specific toxicological effect is not observed in all the animal species investigated. This species difference provides relevant ‘strength and consistency’ arguments in favour of a postulated mode of action. The subsequent assessment of the human relevance of the effect can then be directed at discovering whether the human is similar to the susceptible or to the non-susceptible animal species. This can only be addressed by additional investigations, such as *in vitro* studies with human tissues. This means that once the mode of action in experimental animals is understood, an *in vitro* model system (cellular / sub-cellular) correctly predicting the observed response in susceptible and non-susceptible animals *in vivo*, can be established. As
Toxicological Modes of Action: Relevance for Human Risk Assessment

illustrated in Scheme 3-2, the *in vitro* system used with human samples supports the process of extrapolation to humans.

**Scheme 3-2**

The overall approach described in this section for extrapolation to humans of the lead toxic effect observed in animals is summarised in Scheme 3-3. This lead effect is usually the most sensitive endpoint of those affected adversely by exposure to the test substance. Though an effect may be eliminated on the basis that its mode of action is non-relevant for humans, it cannot be concluded from this that no hazard exists for the substance under consideration. There are two likely scenarios: either a different endpoint should be selected, which will then become the new ‘lead’ effect to be examined as a basis for the assessment of human risk. Alternatively, the endpoint under consideration may still be used in the risk assessment but taking account of the factors that determine the species difference to establish a realistic margin of safety, e.g. based on more sophisticated dosimetry.
1 The Hill criteria may not be met if the postulated mode of action is incorrect or if the available evidence is inadequate to support the hypothesis. In such cases the default factors for species extrapolation should be applied. Alternatively, a decision could be made to develop and test an alternative hypothesis for the mode of action or to conduct additional studies towards meeting the Hill criteria.
4. THE APPROACH IN PRACTICE

The evaluation of the potential for a chemical to adversely affect human health begins with a review of the toxicological data set of which the major part generally comprises the results of experimental studies conducted according to regulatory guidelines. A structured approach for evaluating the outcome of toxicology studies to identify the substance-induced adverse effects is described in ECETOC Technical Report No 85 (ECETOC, 2002). From such studies, where more than one adverse effect arises from exposure to the substance under evaluation, the lead effect is identified and used as the basis for assessment of human risk.

The default assumption made is that the effect is directly relevant to humans and safe levels of exposure are defined by application of assessment factors to the NOAEL established for that effect in the most sensitive species. Further details and guidance on the derivation and application of appropriate assessment factors in human health risk assessment can be found in ECETOC Technical Report No 86 (ECETOC, 2003).

However, where the data indicate that the effect is likely to be of questionable relevance to humans, the following structured process for confirming the decision to depart from the default approach should be followed.

4.1 An established mode of action

Where a ‘new’ substance under investigation appears to be exerting its lead effect via a mode of action that is similar to one that has been well-studied and established as one that is non-relevant to humans, it may be sufficient to confirm the non-relevance of the effect in the ‘new’ substance by examining its potential to evoke one or more of the key events. This approach, summarised in Scheme 4-1, is illustrated by the following examples in which the key events in the toxicological process of well-studied and accepted mechanisms are proposed as sufficient evidence for confirming that a ‘new’ substance would act via the same mechanism of toxicity.
Scheme 4-1

Lead effect of concern evoked by other substances via well-established mode of action

Do published data from other substances suggest that mode of action is not relevant for humans?

YES

Test mode of action hypothesis for the new substance

NO

Do data meet Hill criteria?

YES

Assessment of human relevance not necessary; already established

NO

Apply default for species extrapolation

Risk assessment

Depart from default for species extrapolation. Select different endpoint (new lead effect)

Step 1

Step 2

1 The Hill criteria may not be met if the postulated mode of action is incorrect or if the available evidence is inadequate to support the hypothesis. In such cases the default factors for species extrapolation should be applied. Alternatively, a decision could be made to develop and test an alternative hypothesis for the mode of action or to conduct additional studies towards meeting the Hill criteria.
4.1.1 α2u-Globulin-mediated nephropathy in male rats

Where nephrotoxicity or increased kidney tumour rates occur exclusively in male rats (after subacute / subchronic or long-term treatment respectively), the suspicion should be that these changes are α2u-globulin-mediated. The sequence of critical events for this mode of action is presented in Scheme 4-2.

Scheme 4-2
α2u-Globulin-mediated nephropathy is a highly species- and sex-specific syndrome. No protein similar to α2u-globulin (α2u) has been detected in humans, indicating that humans are not at risk for kidney toxicity and tumour formation on exposure to substances that operate through a α2u mediated mode of action (Olson et al., 1990; Borghoff and Lagarde, 1993). However, the question arises as to which parts of this sequence of events have to be corroborated experimentally to confirm the hypothesis for a ‘new’ substance.

The US EPA (1991a) proposed minimal requirements that included in particular, male rat specific nephrotoxicity and carcinogenicity induced by a non-genotoxic substance. The other requirements included demonstration of an increase in the number and size of protein droplets, identification of α2u as the protein accumulating in the droplet and pathology characteristic of the accumulation of protein.

Studies of only a few days duration would be sufficient to demonstrate the accumulation of protein droplets in the renal tubular cells by immunohistochemistry using specific α2u antibodies. Accordingly, if under these conditions unequivocal evidence establishes that the accumulated protein droplets are essentially α2u, the Task Force believes that no further investigations are needed.

4.1.2 Forestomach tumours

Where long-term oral treatment of rats and/or mice with a non-genotoxic substance has revealed an increased incidence of forestomach tumours, this should immediately be considered rodent-specific, based on the knowledge that the forestomach is not present in humans, primates or carnivores, and the large amount of information on other substances that have induced tumours at this site in rodents. Further supportive evidence can be obtained by a careful analysis of tumour incidences at other locations (which in general should not be increased) as well as non-neoplastic effects in the oesophagus, forestomach and glandular stomach. In this case hyperplastic changes should not be present in the oesophagus and the glandular part of the stomach or those forestomach parts not affected by papillomas / carcinomas.

The sequence of events shown in Scheme 4-3 provides further guidance on confirming the hypothesis of a non-genotoxic, rodent-specific effect.
Scheme 4-3

- **Treatment**
  - Inflammatory response in forestomach epithelia
  - Progressive cytotoxicity
  - Compensatory cell proliferation
  - Epithelial hyperplasia and dysplasia
  - Papilloma
  - Squamous cell carcinoma
According to this scheme, it would be appropriate to conduct a short-term (7-28 days) study in the respective species with routine histology focused on the forestomach (especially limiting ridge), the oesophagus and the glandular stomach. This study could be restricted to the sex showing increased sensitivity to this effect.

If the results of this investigation match the overall picture as described above, no further special investigations should be necessary to confirm that the effect is the result of a mode of action that is not relevant to humans.

4.1.3 Thyroid gland stimulation by enzyme inducers

Various modes of action are known by which chronic exposure of rats to chemical substances results in the formation of thyroid gland tumours.

If one such substance of interest has been shown to be non-genotoxic, then one possible mode of action is through its ability to cause an imbalance in thyroid hormone metabolism. The sequence of events for this mode of action is shown in Scheme 4-4.

Among such non-genotoxic substances are liver enzyme inducers that increase the rate of the hepatic clearance of thyroid hormones. Decreased serum thyroid hormone, due to increased hepatic clearance, results in a compensatory increase in pituitary thyroid stimulating hormone (TSH) that can exert a tumour promoting effect. The ability of a substance to induce liver enzymes, specifically UDP-glucuronosyltransferase activity towards thyroxine (T4) can be measured using liver microsomes from treated rats. In vivo studies can be conducted at the dose and route of exposure that results in tumour induction, to demonstrate induction of liver enzymes coinciding with decreased triiodothyronine (T3) and T4 serum levels and a compensatory increase in the plasma level of TSH (McClain, 1992; McClain et al, 1989). These special investigations will confirm whether or not the substance under study exerts its thyrotoxic effect via this mode of action that is not relevant to humans.
Toxicological Modes of Action: Relevance for Human Risk Assessment

Scheme 4-4

- Treatment
  - Induction of liver microsomal enzymes (thyroxine UDP-glucuronosyltransferase)
  - Decreased serum concentrations of triiodothyronine and thyroxine
  - Increased serum concentration of thyroid stimulating hormones
    - Thyroid gland stimulation (hypertrophy, hyperplasia)
    - Thyroid gland tumours
4.1.4 Tyrosinaemia and corneal opacity in the rat

Reports of ocular opacity in rat studies might suggest that the lesions were caused by the presence of high concentrations of tyrosine, following inhibition of 4-hydroxyphenyl pyruvate dioxygenase (HPPD). This mechanism is displayed in Scheme 4-5. In mice and humans, even under conditions of strong HPPD inhibition, tyrosine concentrations will not increase to levels high enough to induce ocular toxicity and hence, this toxicity observed in the rat is inappropriate for extrapolation to humans.

**Scheme 4-5**

Ophthalmoscopy and histopathology will clearly be critical in determining whether the lesion in question shows the same features as that reported by Robinson (1995). Inflammation of the cornea (which is relatively rare), keratitis, life history and rapid regression of the lesion when treatment is removed, are the distinct features of this lesion.
Triketones induce ocular opacity in rats by causing a severe tyrosinaemia following a highly specific inhibition of the tyrosine catabolic enzyme, HPPD. If another or additional enzyme of the tyrosine catabolic cascade, such as tyrosine aminotransferase (TAT), were to be inhibited even partially by the substance of interest, the severe tyrosinaemia produced could also result in similar corneal lesions. It is important to realise that, if ocular opacity was reported for a substance that causes tyrosinaemia through partial inhibition of TAT, due to the different pathogenesis of the lesion the same biological effect of corneal opacity, could in those circumstances be relevant to humans. It is therefore critical, to demonstrate that HPPD is specifically inhibited (Provan et al, 1999).

Hence, in assessing the weight of evidence that would support the inclusion of a new substance into a generic class of HPPD inhibitors, the species / sex / age differences in response (severity of tyrosinaemia, incidence of corneal opacity) need to be consistent with that reported in the literature for substances where this mechanism is proven. In addition to proving that the corneal opacity is the same as that reported for model HPPD inhibitors, the fundamental cause of the tyrosinaemia must also be clearly elucidated and it should be known if the substance directly affects other enzymes of tyrosine catabolism.

4.1.5 Other modes of action whose human relevance has been questioned

Further examples of generic, non-genotoxic modes of action that may lead to effects in susceptible species but whose relevance to humans has been questioned are given in Table 4-1. Where such effects are observed for a ‘new’ chemical substance, investigation of the critical elements of the mode of action may lead to the conclusion that this aspect of toxicity is not relevant for humans and justify departure from the default approach for assessment of risk.
### Table 4-1: Effects with modes of action specific to susceptible species

<table>
<thead>
<tr>
<th>End point / Effect</th>
<th>Mode of Action</th>
<th>Species / Sex Sensitivity</th>
<th>Type of Difference</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leydig cell tumours</td>
<td>Disruption of hypothalamic-pituitary-testicular axis; resulting in sustained increase in circulatory luteinising hormone and increased incidence of Leydig cell tumours</td>
<td>Rats ++</td>
<td>Toxicodynamic (quantitative)</td>
<td>Clegg et al, 1997; Christensen and Peacock, 1980; Cook et al, 1992, 1997, 1999</td>
</tr>
</tbody>
</table>
### Table 4-1: Effects with modes of action specific to susceptible species (cont’d)

<table>
<thead>
<tr>
<th>End point / Effect</th>
<th>Mode of Action</th>
<th>Species / Sex Sensitivity</th>
<th>Type of Difference</th>
<th>References</th>
</tr>
</thead>
</table>
| Liver tumours      | Induction of hepatic peroxisome proliferation with hepatic cell proliferation; resulting in preneoplastic lesions and eventual tumour formation | Rats ++  
Mice ++  
Guinea pigs -  
Cats -  
Dogs -  
Primates -  
4.2 *An observed effect with an unknown mode of action*

Where a ‘new’ substance under investigation appears to be exerting its lead effect via a mode of action that has not been already described and established as generic for other substances, the default assumption of relevance to humans must be applied unless the data suggest otherwise. In such cases a thorough application of the approach outlined in Section 3 is recommended in order to justify departure from the default extrapolation process. As shown in Scheme 3-3, having identified the lead effect and found evidence to doubt its relevance to humans, e.g. it appears to be species-specific; the first major step to be taken is the elucidation of the mode of action. Once this has been established, the second major step is to evaluate the relevance of this mode of action to humans.

As already emphasised, it is a challenging task to identify and demonstrate conclusively a sequence of key events for a new type of effect, and to establish within this sequence at least one event that will not take place in humans. In order to achieve this goal convincingly, in most cases, extensive additional experimentation will be unavoidable.

Coumarin, methylene chloride and formaldehyde are examples of substances which have undergone extensive investigation to elucidate their mechanism and mode of action to demonstrate non-relevance of the findings to humans; their case summaries are given in Appendix A.
5. CONCLUSION

The assessment of hazard and risk to humans that might arise from exposure to a chemical substance relies predominantly on the extrapolation of data generated in studies with experimental animals. The objective of such risk assessments should be to describe, with as little uncertainty as possible, the potential for unwanted health effects arising in humans due to their exposure to potentially hazardous chemicals. In practical terms, the assumption is made that the adverse effects evoked by the substance in various regulatory tests would also be expressed in humans and the risk assessment based on the most critical or ‘lead’ effect in the most sensitive animal species. To account for uncertainties including the possibility of greater sensitivity of humans to the effects of the chemical, assessment factors are applied in deriving ‘safe’ exposures for humans.

For a significant number of substances, it has been shown that adverse effects observed in animal studies would not be manifest in humans and would therefore not be a relevant basis for hazard or risk assessment. It is generally accepted that the expression of toxicity in a mammalian system is dependent on a sequence of key events taking place, each of which is critical for the ultimate effect to be observed. Thus, for a specific toxic endpoint, to justify deviating from the default approach in risk assessment, it should be sufficient to identify the key events in the process (mode of action) and establish that at least one of them would not occur in humans, or would occur only to a much lesser extent. From such knowledge it could be concluded that the toxic outcome would not be observed in humans, or be observed only at much higher and possibly irrelevant exposure levels.

A number of these cases are described in this report and used to develop a structured approach to justify departure from the use of default assessment factors. In evaluating a ‘new’ chemical substance, having identified the lead effect from the toxicology data set, before proceeding with the default approach, the experimental animal data should be examined to establish whether:

a) the effect of the chemical to be assessed differs qualitatively or quantitatively in different test species;

b) the effect of the chemical to be assessed is generically similar to other compounds with a well-established mode of action that is not considered relevant to humans.

In the case of either or both, it would be appropriate to proceed by analysing the data by applying the two-step approach detailed in Chapters 3 and 4, namely:

1. to identify the mode of action of toxicity in the susceptible species;
2. to assess the relevance of the identified mode of action for humans.
Where this analysis confirms a qualitative difference in the sensitivity between the experimental animals and humans to the toxic effect, this endpoint should not be used in assessing human risk. The toxicity database should be examined to identify the next most sensitive endpoint or lead effect (relevant to humans) to be used as a basis for the assessment. Chronic nephropathy and subsequent kidney tumour formation in the male rat via α2u and forestomach tumour formation in rodents are examples where a qualitative difference has been demonstrated convincingly.

Where it is shown that the difference between animals and humans in expression of the adverse effect is quantitative, the evaluation of human hazard and risk must be undertaken using a case by case approach. This is because the outcome will depend on the extent of the difference, i.e. it will determine whether it would be appropriate to select a different sensitive endpoint or whether it would justify the derivation of more appropriate risk estimates for humans in place of applying the default assessment factors. An example of such is formaldehyde, where the risk assessment for respiratory tract effects was based on use of biomarkers in the target tissues to establish a realistic margin of safety.

Where the evidence supports the case for species differences in sensitivity, qualitative or quantitative, which have not been investigated or described for other substances, the process for establishing the non-relevance to humans of that effect is likely to require extensive additional investigations.

The use of this approach to identify those substances, which express toxicity in experimental animals via a mode of action that is not relevant to humans, will enable their more realistic risk assessment. Furthermore, adopting a ‘mode of action’ approach should considerably speed up the risk assessment process and avoid unnecessary animal experimentation.
# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>2,4-D</td>
<td>2,4-Dichlorophenoxyacetic acid</td>
</tr>
<tr>
<td>2,4,5-T</td>
<td>2,4,5-Trichlorophenoxyacetic acid</td>
</tr>
<tr>
<td>α-KG</td>
<td>α-Ketoglutarate</td>
</tr>
<tr>
<td>α2u</td>
<td>α2u-Globulin</td>
</tr>
<tr>
<td>α2u-N</td>
<td>α2u-Globulin nephropathy</td>
</tr>
<tr>
<td>ADME</td>
<td>Absorption, distribution, metabolism and excretion</td>
</tr>
<tr>
<td>AIHC</td>
<td>American Industry Health Council</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under curve</td>
</tr>
</tbody>
</table>
| CIIT         | Formerly: Chemical Industry Institute of Toxicology  
               Now: Centers for Health Research |
| DNA          | Deoxyribonucleic acid |
| EC           | European Community |
| EU           | European Union |
| HPPD         | 4-Hydroxyphenyl pyruvate dioxygenase |
| IARC         | International Agency for Research on Cancer |
| JMAFF        | The Japanese Ministry of Agriculture, Forestry and Fisheries |
| MCPA         | 4-Chloro-2-methylphenoxyacetic acid |
| NOAEL        | No observed adverse effect level |
| NTBC         | 2-(2-Nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione |
| T3           | Triiodothyronine |
| T4           | Thyroxine |
| TAT          | Tyrosine aminotransferase |
| TRH          | Thyrotropin-releasing hormone |
| TSH          | Thyroid stimulating hormone |
| UDP          | Uridine diphosphate |
| US EPA       | US Environmental Protection Agency |
| WHO          | World Health Organization |
APPENDIX A: TOXICOKINETIC DIFFERENCES BETWEEN ANIMALS AND HUMANS

The following examples are well-documented in the literature, but for ease of reference details are provided below.

**Coumarin**

Coumarin is used clinically in the treatment of high-protein oedema and has been tested in several clinical trials for the treatment of certain malignancies. Due to its use as a drug, information is available concerning its toxicity and metabolism in humans.

The liver is the major target organ for toxicity and carcinogenicity of coumarin in rats (National Toxicology Program, 1993). At levels that exceeded the maximum tolerated dose (5000 ppm), chronic dietary administration of coumarin resulted in increased incidences of cholangiocarcinomas and parenchymal liver cell tumours (Carlton *et al*, 1996). Other species, such as the Syrian hamster and the baboon, are much more resistant to coumarin-induced toxicity (Evans *et al*, 1979). In the Syrian hamster, subchronic and/or chronic administration failed to induce vacuolar degeneration, apoptosis, bile duct degeneration or sustained stimulation of DNA synthesis (Lake *et al*, 1990; Lake and Grasso, 1996; Ueno and Hirono, 1981). No increased incidence of liver tumours was observed in the chronic studies. Baboons fed at the maximum tolerated dose for up to 2 years showed no evidence of biliary hyperplasia or fibrosis, and lesions were limited to some ultrastructural changes compatible with early cell damage (Evans *et al*, 1979).

In rats, the main route of coumarin metabolism is via oxidation to coumarin 3,4-epoxide and only minor amounts of 7-hydroxylation metabolites are formed and excreted (Lake, 1999). Coumarin 3,4-epoxide is rapidly metabolised to o-hydroxyphenylacetaldehyde (Ratanasavanh *et al*, 1996; Born *et al*, 1997); and this metabolite, together with the epoxide, is probably responsible for the toxicity and carcinogenicity in this species. In *vitro* studies have confirmed that the formation of o-hydroxyphenylacetaldehyde is the predominant pathway in Fischer 344 rats (Born *et al*, 2000). In contrast to coumarin, dihydrocoumarin (which lacks the 3,4-double bond), as well as some coumarin metabolites (such as 3- and 7-hydroxycoumarin), produce little toxicity *in vivo* or *in vitro* in rat hepatocytes (Lake *et al*, 1989).

In other species such as the gerbil (Lake *et al*, 1992), Syrian hamster (Lake *et al*, 1990) or baboon (Evans *et al*, 1979), the 7-hydroxylation pathway is predominant, although the ratio between this pathway and 3,4-epoxidation may depend on the dose administered (Born *et al*, 2000).
In humans, coumarin metabolism occurs predominantly via 7-hydroxylation (Egan et al., 1990). Studies with microsomes from 12 different human subjects demonstrated that, even in clinical trials where large dosages of coumarin are administered, epoxidation contributes little to the elimination of coumarin (Born et al., 2000). Little evidence of hepatotoxicity was reported in humans given coumarin in clinical trials to treat various malignancies and chronic infections (Cox et al., 1989; Dexeus et al., 1990).

The available experimental evidence suggests that the predominance of the 7-hydroxylation detoxification pathway significantly contributes to the absence of hepatotoxicity and liver carcinogenicity in the Syrian hamster and in primates including humans (Lake, 1999). The hepatotoxicity and liver carcinogenicity findings in the rat are most likely related to the formation of a reactive epoxide metabolite and o-hydroxyphenylacetalddehyde.

The data available for coumarin suggest that the hepatotoxicity and liver carcinogenicity in rats is inappropriate for extrapolation to humans.

**Methylene chloride**

Liver and lung tumours were observed exclusively in mice on long-term exposure to methylene chloride (Mennear et al., 1988). The carcinogenic mode of action comprises its metabolism to a reactive glutathione conjugate, followed by covalent binding of this conjugate to DNA, leading ultimately to cancer (Green, 1997). DNA-protein cross-linking as a consequence of formaldehyde formation from the reactive glutathione metabolite may also contribute to tumour formation. In the lung, cytotoxicity (to Clara cells) and increased cell proliferation were probable contributory factors (Burek et al., 1984). The species specificity of tumour formation is a consequence of the presence of a theta-glutathione S-transferase with a high activity and cellular as well as nuclear localisation, which seems to be unique to the mouse. In other species including the rat, hamster and humans, the glutathione conjugate pathway is much less important (Green, 1997). Although the high nuclear enzyme activity in the mouse resulted in DNA damage in liver and lung cells, DNA damage could not be detected *in vitro* in hepatocytes from hamsters and human. In rat hepatocytes, DNA damage was only seen at high concentrations of methylene chloride that could not be achieved *in vivo*, thus explaining the lack of carcinogenicity in the rat (Graves et al., 1995).

The data available for methylene chloride suggest that the carcinogenic response in the mouse is unique for this species and inappropriate for extrapolation to humans.
**Formaldehyde**

Inhalation bioassays with formaldehyde revealed nasal squamous cell carcinoma in rats and mice (Kerns et al., 1983; Albert et al., 1982; Feron et al., 1988; Woutersen et al., 1989). Formaldehyde causes mutations and DNA damage in bacteria. It induces gene mutations, chromosomal aberrations, sister chromatid exchanges, DNA strand breaks, and DNA-protein cross-links in mammalian cells (IARC, 1995a). The induction of mutations by formaldehyde is dependent on the rate of cell replication. High rates of cell replication are a normal tissue response to cytolethality and are induced in the rat nasal epithelium by high concentrations of formaldehyde (Monticello et al., 1996). Tumour formation is only observed at dose levels that cause tissue damage and regenerative cell proliferation. DNA-protein cross-links are formed when inhaled formaldehyde reaches the nuclei of nasal epithelial cells (Casanova et al., 1994). Enhanced cell replication in the presence of DNA-protein cross-links is proposed to be the mode of action. The rate of protein-DNA cross-link formation is directly related to the concentration of free formaldehyde in nasal epithelial cell nuclei. Thus, DNA-protein cross-links provide a biomarker for effective target tissue dose of formaldehyde (Casanova et al., 1989; Casanova et al., 1991; Casanova et al., 1994; Heck et al., 1990). DNA-protein cross-link measurements in rats and monkeys showed steep exposure-biomarker relationships in both species (Casanova-Schmitz et al., 1984; Casanova et al., 1989; Casanova et al., 1991; Casanova et al., 1994). Physiologically based kinetic modelling using rat data and incorporating species differences in physiological parameters allowed the correct prediction of DNA-protein cross-link values for monkeys (Casanova et al., 1991; Casanova et al., 1994). Because of similarity of the monkey nasal anatomy and air flow characteristics to the human, it is assumed to be the more appropriate species for estimating human risk.

The formaldehyde example demonstrates the usefulness of dose-response information for a key precursor event for risk assessment (Haber et al., 2001). DNA-protein cross-links as a target tissue dosimeter in place of inhaled formaldehyde concentration allowed more sophisticated risk assessment which resulted in lower risk estimates for humans (US EPA, 1991b; California Air Resources Board, 1992; CIIT, 1999).

**Phenoxyacetic acids**

A number of experimental studies have demonstrated that the dog is particularly susceptible to the adverse effects of the weak organic acids 2,4-dichlorophenoxyacetic acid (2,4-D), 4-chloro-2-methylphenoxyacetic acid (MCPA) and the related herbicides triclopyr and 3,5,6-trichloro-2-pyrinyloxyacetic acid. (Arnold and Beasley, 1989; Gehring and Betso, 1978). As a class, these compounds are known to have similar pharmacokinetic properties.
The kinetics and metabolism of MCPA were investigated in rats and dogs after oral dosing with (14C)-MCPA at 5 mg kg⁻¹ (Lappin et al., 2002). Radioactivity was eliminated from plasma significantly more quickly in the rat than in the dog, resulting in a more than 10-fold higher AUC (area under the curve) value (based on total radioactivity) in dog compared with rat.

The toxicological profile of 2,4-D is much like that of MCPA. A toxicokinetic study designed to investigate whether similar conclusions could be drawn for 2,4-D (van Ravenzwaay et al., 2003) showed that elimination of the radioactive dose of 2,4-D from rat plasma was significantly more rapid than in the dog. The approximate half-life values were 1.3-3.4 h for the rat and 99-134 h for the dog following a 5 or 50 mg kg⁻¹ dose, respectively. Comparison of the plasma AUC showed that the values for dog were 27 (high dose) to 232 (low dose) times higher than the corresponding values in the rat. These results showing higher systemic exposure to the test material in the dog are consistent with the increased sensitivity of this species to 2,4-D toxicity.

Similar compounds such as dichlorprop and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) are known to be excreted by active renal mechanisms (Beitz et al., 1985). Basolateral accumulation of 2,4-D has been shown to be mediated by 2,4-D/α-ketoglutarate (α-KG) exchange, coupled indirectly to Na⁺/α-KG co-transport in the same way as ρ-aminohippurate and fluorescein (Villalobos et al., 1996). Moreover, in in vitro studies with kidney slices, 2,4-D was shown to concentrate in the kidney tissue through an active transport mechanism (Berndt and Koschier, 1973).

Investigations by Timchalk and Nolan, 1997; van Ravenzwaay et al., 2003; and Lappin et al., 2002 show clearly that dogs, in contrast to rats, are deficient in their ability to excrete a range of organic acids. At least for 2,4-D, metabolism is required in the dog before the compound can be excreted (van Ravenzwaay et al., 2003). In rats, monkeys or in humans, metabolism does not seem to be a prerequisite for excretion (Timchalk and Nolan, 1997).

Comparative pharmacokinetics of phenoxyacetic acids and structurally related organic acids in a variety of species including humans were evaluated using allometric parameter scaling (Timchalk, 2004). For both 2,4-D and MCPA, the dog plasma half-life and renal clearance rates could not be reasonably scaled across species. For all other species evaluated, including humans, scaling of the same pharmacokinetic parameters was possible. These findings demonstrate that the dog is not an appropriate model to investigate the toxicity of phenoxyacetic and other structurally related organic acids for humans. The European Union also concluded that for 2,4-D, the dog was not the relevant species for setting of regulatory values for human health risks and that these should be based on a NOAEL from rodent studies (EU, 2001).
APPENDIX B: TOXICODYNAMIC DIFFERENCES BETWEEN ANIMALS AND HUMANS

α2u-Globulin-mediated kidney tumours in male rats

Chemically-induced α2u-globulin nephropathy (α2u-N) occurs in male rats following exposure to a variety of non-genotoxic substances (Borghoff et al, 1990; Hard et al, 1993; Swenberg and Lehman-McKeeman, 1999). Chronic exposure of male rats to such substances induces a low incidence of renal tumours in male rats. Following exposure in the male rat, the specific low molecular weight protein α2u-globulin (α2u) accumulates in the form of protein droplets in proximal tubule epithelial cells. Normally, α2u is synthesised in the male rat liver, secreted into blood and filtered at the glomerulus, with approximately 50-60% reabsorbed into kidney proximal tubular cells under physiological conditions (Roy and Neuhaus, 1966; Neuhaus, 1986; MacInnes et al, 1986). Substances that cause α2u-N bind reversibly to α2u (Swenberg and Lehman-McKeeman, 1999; Prescott-Matthews et al, 1999) and, in addition, increase the rate of direct uptake of α2u into lysosomes of proximal tubular cells (Cuervo et al, 1999). The chemical-α2u complex is more resistant to hydrolytic degradation that leads to its accumulation as protein droplets (Lehman-McKeeman et al, 1990). Cytolality occurs when this accumulation reaches a certain level. As a result of cell death and degeneration, there is compensatory cell proliferation in the region where the α2u accumulates (Short et al, 1987). This increase in cell proliferation enhances the likelihood of spontaneous mutational events and may result in the clonal expansion of initiated cells leading to tumour formation (Butterworth et al, 1992).

α2u-N is a highly species- and sex-specific syndrome. No protein similar to α2u has been detected in humans, indicating that humans are not at risk for kidney toxicity and tumour formation on exposure to substances that operate through an α2u mediated mode of action (Olson et al, 1990; Borghoff and Lagarde, 1993).

Forestomach tumours

In rodents the stratified squamous epithelium of the oesophagus continues into the cardiac glandular portion of the stomach to form the covering of the forestomach. These stomach structures are not present in humans, monkeys, dogs, or guinea pigs (Grice, 1988). The rodent forestomach is the target organ for a large number of non-genotoxic as well as genotoxic substances (Kroes and Wester, 1986). Exposure to relatively high oral doses of certain non-genotoxic substances triggers a typical sequence of morphological changes. Initially, cellular proliferation leads to hyperplasia and, if exposure is continued, to papillomas and finally to squamous cell carcinomas (Clayson et al, 1991; Iverson, 1995). If treatment is stopped at a phase
where tumours have not yet developed, these changes are fully reversible (Altmann et al., 1985; Hirose et al., 1990; Kagawa et al., 1993). The oesophagus and the glandular part of the stomach are not usually affected (Ito et al., 1986; Wester and Kroes, 1988). Stimulation of cell division resulting in cell proliferation in the forestomach epithelium occurs within only a few days if high dosages are administered (Clayson et al., 1991; Iverson, 1995; Lutz et al., 1997; Verhagen et al., 1988). Cell proliferation is greatest in the squamous epithelium immediately adjacent to the glandular mucosa (i.e. the limiting ridge), and this is also the site at which most tumours develop (Powell and Berry, 1999). The link between cellular proliferation and the development of forestomach tumours has not yet been fully elucidated; nevertheless sustained stimulation of cell proliferation can lead to tumour formation (Clayson et al., 1991). There is a clear-cut threshold; dose levels which do not cause morphological changes (such as cell proliferation or hyperplasia) are not tumorigenic.

In species, including humans, that lack a forestomach comparable to rodents, the sequence of events described above cannot take place (Grice, 1988; Moch, 1988). Moreover, the oesophagus (which cytologically resembles the forestomach epithelium), is not a target tissue either in rodents or non-rodents for tumour formation from such substances (Kroes and Wester, 1986). Consequently, there is no tumorigenic / carcinogenic potential for humans.

**Thyroid gland stimulation by enzyme inducers**

Thyroid cancer incidence in humans is low, while thyroid neoplasia is common in rodent, especially rat carcinogenicity studies. This is explained by two major differences between rodent and human thyroid physiology: (1) absence of a high affinity thyroid hormone-binding protein in rodents, with the consequence of much shorter plasma half-life of thyroid hormones in these species and (2) sustained stimulation of the thyroid gland by increased plasma levels of thyroid stimulating hormone (TSH) is associated with thyroid follicular cell neoplasia in rodents but not in humans (Curran and DeGroot, 1991).

Homeostasis of thyroid hormone synthesis and secretion is normally controlled by a sensitive feedback mechanism that involves the hypothalamus, the pituitary and the thyroid gland (Capen et al., 1991; Paynter et al., 1988, Owen et al., 1973; Atterwill and Brown, 1988). Of particular importance in this feedback mechanism is TSH, which is secreted by the pituitary and stimulates the thyroid gland to synthesise and release thyroid hormones. The rate of TSH release is controlled by the amount of thyrotropin-releasing hormone (TRH) released by the hypothalamus as well as by circulating triiodothyronine (T3) and thyroxine (T4) levels. When circulating T3 and T4 levels are reduced, the pituitary is triggered to secrete TSH, which in turn stimulates the thyroid to produce T4 and T3. The thyroid produces predominantly T4 which is either 5’-deiodinated to give the more active hormone T3 or undergoes inner ring deiodination to form rT3.
which has no known hormonal activity. Apart from T4 deiodination, degradation of thyroid hormones occurs mainly in the liver where both T4 and T3 are conjugated to the respective glucuronide or sulphate that are then excreted via bile into the intestine (Hill et al., 1989; van Raaij et al., 1993).

Substances that induce xenobiotic hepatic metabolising enzymes can increase the plasma clearance of thyroid hormones; for example, increased hepatic glucuronidation rate of T4 as a consequence of thyroxine UDP-glucuronosyltransferase induction (Capen et al., 1991; McClain et al., 1989; McClain, 1992). The absence of specific plasma binding proteins for thyroid hormones renders rodents more susceptible to increased rates of thyroid hormone removal from blood. As a feedback response, increased plasma TSH levels will stimulate the gland, accelerate thyroid hormone production, and increase DNA synthesis as well as cell proliferation (Hill et al., 1989; Capen et al., 1991). On chronic exposure, nodular hyperplasia and finally follicular adenomas and carcinomas are formed (McClain et al., 1989).

Despite a common physiology between rodents and humans, humans are much more resistant to chemical disturbances of the thyroid-pituitary axis and do not develop cancer from its chemically-induced perturbation (McConnell, 1992; Capen et al., 1991; Capen, 1992; McClain, 1992).

**Triketone-mediated tyrosinaemia and corneal opacity**

Various triketones, including 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC) and mesotrione, have been reported to cause corneal opacity in rats but not in mice (Lock et al., 1996, 2000). Investigative toxicology studies have revealed that this species-specific ocular toxicity is caused by the action of high concentrations of tyrosine at the target site, rather than by the direct action of xenobiotics, or metabolites thereof, on the eye (Robinson 1995; Lock et al., 1996; Ellis et al., 1995).

The basis for the species difference in expression of toxicity is due to differences in the disposition of tyrosine, the ultimate toxiphore, between rats and mice (Provan et al., 1999). The rate-limiting step in the removal of excess tyrosine is its conversion to 4-hydroxyphenylpyruvate by tyrosine aminotransferase (TAT) (Goldsmith, 1983). Rats have much lower activity of hepatic TAT than mice and are therefore unable to prevent the build up of high and toxic tyrosine levels in plasma (Provan et al., 1999). In contrast, mouse hepatic TAT activity is of sufficient magnitude to prevent tyrosine concentrations of reaching toxic levels.
Data indicate the activity of hepatic TAT in humans to be similar to mice (Henderson et al., 1981), suggesting that the mouse is a more appropriate model than the rat to investigate the potential hazard of mesotrione-induced ocular toxicity in humans.

NTBC is approximately 1000-fold more potent than mesotrione in its inhibition of 4-hydroxyphenyl pyruvate dioxygenase (HPPD) (Hall et al., 2001). For NTBC, which is used internationally in the treatment of children suffering from the rare hereditary disease Tyrosinaemia Type I, there is currently over 1000 patient-years clinical experience without evidence for adverse ocular effects (personal communication with Swedish Orphan AB). Furthermore studies have been conducted with NTBC and mesotrione in healthy male volunteers (Hall et al., 2001). With mesotrione, only marginally increased plasma tyrosine concentrations were observed which quickly returned to control levels. Steady state plasma tyrosine concentrations with NTBC were also much lower than those observed in the rat. No ocular toxicity was associated on human exposure to either NTBC or mesotrione.

The corn herbicide 2-(4-methylsulfonyl-2-nitrobenzoyl-1,3-cyclohexanedione (mesotrione) and other triketones, such as NTBC are inhibitors of HPPD (Ellis et al., 1995; Provan et al., 1999). In mammals, HPPD is the second enzyme of the catabolic cascade for removal of excess dietary tyrosine. The biological consequence of HPPD inhibition in mammals has been shown to be a rise in plasma tyrosine.

In mice and humans, even under conditions of strong HPPD inhibition, tyrosine concentrations will not increase to levels high enough to induce ocular toxicity and hence, this toxicity observed in the rat is inappropriate for extrapolation to humans.
Toxicological Modes of Action: Relevance for Human Risk Assessment

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<td>Head, Environmental Safety Department</td>
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<td>Senior associate, Medical Adviser</td>
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<td>(now Arkema group)</td>
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</tbody>
</table>
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*Stewards: Dr. N. Carmichael (Bayer CropScience); resigned from the Scientific Committee on 31.12.2003.
Dr. A. Sarrif (DuPont); resigned from the Scientific Committee on 31.12.2004.
Dr. B. van Ravenzwaay acted as Steward for publication.
## ECETOC PUBLISHED REPORTS

### Monographs

<table>
<thead>
<tr>
<th>No.</th>
<th>Title</th>
<th>Published Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. 1</td>
<td>Good Laboratory Practice</td>
<td>Published October 1979</td>
</tr>
<tr>
<td>No. 2</td>
<td>A Contribution to Strategy for Identification and Control of Occupational Carcinogens</td>
<td>Published September 1980</td>
</tr>
<tr>
<td>No. 3</td>
<td>Risk Assessment of Occupational Chemical Carcinogens</td>
<td>Published May 1985</td>
</tr>
<tr>
<td>No. 4</td>
<td>Hepatocarcinogenesis in Laboratory Rodents: Relevance for Man</td>
<td>Published October 1982</td>
</tr>
<tr>
<td>No. 5</td>
<td>Identification and Assessment of the Effects of Chemicals on Reproduction and Development (Reproductive Toxicology)</td>
<td>Published December 1983</td>
</tr>
<tr>
<td>No. 6</td>
<td>Acute Toxicity Tests, LD&lt;sub&gt;50&lt;/sub&gt; (LC&lt;sub&gt;50&lt;/sub&gt;) Determinations and Alternatives</td>
<td>Published May 1985</td>
</tr>
<tr>
<td>No. 7</td>
<td>Recommendations for the Harmonisation of International Guidelines for Toxicity Studies</td>
<td>Published December 1985</td>
</tr>
<tr>
<td>No. 8</td>
<td>Structure-Activity Relationships in Toxicology and Ecotoxicology: An Assessment (Summary)</td>
<td>Published June 1986</td>
</tr>
<tr>
<td>No. 9</td>
<td>Assessment of Mutagenicity of Industrial and Plant Protection Chemicals</td>
<td>Published June 1987</td>
</tr>
<tr>
<td>No. 10</td>
<td>Identification of Immunotoxic Effects of Chemicals and Assessment of their Relevance to Man</td>
<td>Published August 1987</td>
</tr>
<tr>
<td>No. 11</td>
<td>Eye Irritation Testing</td>
<td>Published June 1988</td>
</tr>
<tr>
<td>No. 12</td>
<td>Alternative Approaches for the Assessment of Reproductive Toxicity (with emphasis on embryotoxicity/teratogenicity)</td>
<td>Published November 1989</td>
</tr>
<tr>
<td>No. 13</td>
<td>DNA and Protein Adducts: Evaluation of their Use in Exposure Monitoring and Risk Assessment</td>
<td>Published October 1989</td>
</tr>
<tr>
<td>No. 14</td>
<td>Skin Sensitisation Testing</td>
<td>Published March 1990</td>
</tr>
<tr>
<td>No. 15</td>
<td>Skin Irritation</td>
<td>Published July 1990</td>
</tr>
<tr>
<td>No. 16</td>
<td>Early Indicators of Non-Genotoxic Carcinogenesis</td>
<td>Published June 1991</td>
</tr>
<tr>
<td>No. 17</td>
<td>Hepatic Peroxisome Proliferation</td>
<td>Published May 1992</td>
</tr>
<tr>
<td>No. 18</td>
<td>Evaluation of the Neurotoxic Potential of Chemicals</td>
<td>Published September 1992</td>
</tr>
<tr>
<td>No. 19</td>
<td>Respiratory Allergy</td>
<td>Published August 1993</td>
</tr>
<tr>
<td>No. 20</td>
<td>Percutaneous Absorption</td>
<td>Published August 1993</td>
</tr>
<tr>
<td>No. 21</td>
<td>Immunoxicity: Hazard Identification and Risk Characterisation</td>
<td>Published September 1994</td>
</tr>
<tr>
<td>No. 22</td>
<td>Evaluation of Chemicals for Oculotoxicity</td>
<td>Published November 1994</td>
</tr>
<tr>
<td>No. 23</td>
<td>Receptor Mediated Mechanisms in Chemical Carcinogenesis</td>
<td>Published December 1995</td>
</tr>
<tr>
<td>No. 24</td>
<td>Risk Assessment for Carcinogens</td>
<td>Published July 1996</td>
</tr>
<tr>
<td>No. 25</td>
<td>Practical Concepts for Dose Selection in Chronic Toxicity and Carcinogenicity Studies in Rodents</td>
<td>Published February 1996</td>
</tr>
<tr>
<td>No. 26</td>
<td>Aquatic Toxicity Testing of Sparingly Soluble Volatile and Unstable Substances</td>
<td>Published September 1996</td>
</tr>
<tr>
<td>No. 27</td>
<td>Aneuploidy</td>
<td>Published August 1997</td>
</tr>
<tr>
<td>No. 28</td>
<td>Dose-response and threshold-mediated mechanisms in mutagenesis - Mutation Research Special Issue</td>
<td>Published January 2000</td>
</tr>
<tr>
<td>No. 29</td>
<td>Skin Sensitisation Testing for the Purpose of Hazard Identification and Risk Assessment</td>
<td>Published September 2000</td>
</tr>
<tr>
<td>No. 30</td>
<td>Genetic Susceptibility to Environmental Toxicants</td>
<td>Published October 2001</td>
</tr>
<tr>
<td>No. 31</td>
<td>Guidance on Evaluation of Reproductive Toxicity Data</td>
<td>Published February 2002</td>
</tr>
<tr>
<td>No. 32</td>
<td>Use of Human Data in Hazard Classification for Irritation and Sensitisation</td>
<td>Published July 2002</td>
</tr>
<tr>
<td>No. 33</td>
<td>Application of Physiological - Toxicokinetic Modelling to Health Hazard Assessment of Chemical Substances</td>
<td>Published February 2003</td>
</tr>
<tr>
<td>No. 34</td>
<td>Toxicogenomics in Genetic Toxicology and Hazard Determination</td>
<td>Published July 2005</td>
</tr>
</tbody>
</table>
**Technical Reports**

<table>
<thead>
<tr>
<th>No.</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. 1</td>
<td>Assessment of Data on the Effects of Formaldehyde on Humans (updated by TR No. 6) (Published January 1979)</td>
</tr>
<tr>
<td>No. 2</td>
<td>The Mutagenic and Carcinogenic Potential of Formaldehyde (Published May 1981)</td>
</tr>
<tr>
<td>No. 3</td>
<td>Assessment of Test Methods for Photodegradation of Chemicals in the Environment (Published August 1981)</td>
</tr>
<tr>
<td>No. 4</td>
<td>The Toxicology of Ethylene Glycol Monoalkyl Ethers and its Relevance to Man (updated by TR No. 17) (Published June 1982)</td>
</tr>
<tr>
<td>No. 5</td>
<td>Toxicity of Ethylene Oxide and its Relevance to Man (Published September 1982)</td>
</tr>
<tr>
<td>No. 6</td>
<td>Formaldehyde Toxicology: An Up-Dating of ECETOC Technical Reports 1 and 2 (Published September 1982)</td>
</tr>
<tr>
<td>No. 7</td>
<td>Experimental Assessment of the Phototransformation of Chemicals in the Atmosphere (Published September 1983)</td>
</tr>
<tr>
<td>No. 8</td>
<td>Biodegradation Testing: An Assessment of the Present Status (Published November 1983)</td>
</tr>
<tr>
<td>No. 9</td>
<td>Assessment of Reverse-Phase Chromatographic Methods for Determining Partition Coefficients (Published December 1983)</td>
</tr>
<tr>
<td>No. 10</td>
<td>Considerations Regarding the Extrapolation of Biological Data in Deriving Occupational Exposure Limits (Published February 1984)</td>
</tr>
<tr>
<td>No. 11</td>
<td>Ethylene Oxide Toxicology and its Relevance to Man: An Up-Dating of ECETOC Technical Report No. 5 (Published March 1984)</td>
</tr>
<tr>
<td>No. 12</td>
<td>The Phototransformation of Chemicals in Water: Results of a Ring-Test (Published June 1984)</td>
</tr>
<tr>
<td>No. 15</td>
<td>The Use of Physical-Chemical Properties in the 6th Amendment and their Required Precision, Accuracy and Limiting Values (Published June 1984)</td>
</tr>
<tr>
<td>No. 16</td>
<td>A Review of Recent Literature on the Toxicology of Benzene (Published December 1984)</td>
</tr>
<tr>
<td>No. 17</td>
<td>The Toxicology of Glycol Ethers and its Relevance to Man: An Up-Dating of ECETOC Technical Report No. 4 (updated by TR No. 64) (Published April 1985)</td>
</tr>
<tr>
<td>No. 18</td>
<td>Harmonisation of Ready Biodegradability Tests (Published April 1985)</td>
</tr>
<tr>
<td>No. 19</td>
<td>An Assessment of Occurrence and Effects of Dialkyl-o-Phthalates in the Environment (Published May 1985)</td>
</tr>
<tr>
<td>No. 20</td>
<td>Biodegradation Tests for Poorly-Soluble Compounds (Published February 1986)</td>
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<tr>
<td>No. 21</td>
<td>Guide to the Classification of Carcinogens, Mutagens, and Teratogens under the 6th Amendment (Published February 1986)</td>
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<tr>
<td>No. 22</td>
<td>Classification of Dangerous Substances and Pesticides in the EEC Directives. A Proposed Revision of Criteria for Inhalational Toxicity (Published January 1987)</td>
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<tr>
<td>No. 23</td>
<td>Evaluation of the Toxicity of Substances to be Assessed for Biodegradability (Published November 1986)</td>
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<tr>
<td>No. 24</td>
<td>The EEC 6th Amendment: Prolonged Fish Toxicity Tests (Published October 1986)</td>
</tr>
<tr>
<td>No. 25</td>
<td>Evaluation of Fish Tainting (Published January 1987)</td>
</tr>
<tr>
<td>No. 26</td>
<td>The Assessment of Carcinogenic Hazard for Human Beings exposed to Methylene Chloride (Published January 1987)</td>
</tr>
<tr>
<td>No. 27</td>
<td>Nitrate and Drinking Water (Published January 1988)</td>
</tr>
<tr>
<td>No. 28</td>
<td>Evaluation of Anaerobic Biodegradation (Published June 1988)</td>
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<tr>
<td>No. 29</td>
<td>Concentrations of Industrial Organic Chemicals Measured in the Environment: The Influence of Physico-Chemical Properties, Tonnage and Use Patterns (Published June 1988)</td>
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<tr>
<td>No. 30</td>
<td>Existing Chemicals: Literature Reviews and Evaluations (Fifth Edition) (No longer available) (Published May 1994)</td>
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<tr>
<td>No. 31</td>
<td>The Mutagenicity and Carcinogenicity of Vinyl Chloride: A Historical Review and Assessment (Published July 1988)</td>
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<td>No. 32</td>
<td>Methylene Chloride ( Dichloromethane): Human Risk Assessment Using Experimental Animal Data (Published May 1988)</td>
</tr>
</tbody>
</table>
No. 33  Nickel and Nickel Compounds: Review of Toxicology and Epidemiology with Special Reference to Carcinogenesis 
(Published February 1989)

No. 34  Methylene Chloride (Dichloromethane): An Overview of Experimental Work Investigating Species Differences in Carcinogenicity and their Relevance to Man (Published March 1989)

No. 35  Fate, Behaviour and Toxicity of Organic Chemicals Associated with Sediments (Published January 1990)

No. 36  Biomonitoring of Industrial Effluents (Published April 1990)

No. 37  Tetrachlorethylene: Assessment of Human Carcinogenic Hazard (Published May 1990)

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No. 39  Hazard Assessment of Floating Chemicals After an Accidental Spill at Sea (Published July 1990)

No. 40  Hazard Assessment of Chemical Contaminants in Soil (Published April 1992)

No. 41  Human Exposure to N-Nitrosamines, their Effects and a Risk Assessment for N-Nitrosodiethanolamine in Personal Care Products (Published August 1990)

No. 42  Critical Evaluation of Methods for the Determination of N-Nitrosamines in Personal Care and Household Products (Published February 1991)

No. 43  Emergency Exposure Indices for Industrial Chemicals (Published March 1991)

No. 44  Biodegradation Kinetics (Published September 1991)

No. 45  Nickel, Cobalt and Chromium in Consumer Products: Allergic Contact Dermatitis (Published March 1992)

No. 46  EC 7th Amendment: Role of Mammalian Toxicokinetic and Metabolic Studies in the Toxicological Assessment of Industrial Chemicals (Published May 1992)

No. 47  EC 7th Amendment "Toxic to Reproduction": Guidance on Classification (Published August 1992)

No. 48  Eye Irritation: Reference Chemicals Data Bank (Second Edition) (Published June 1998)

No. 49  Exposure of Man to Dioxins: A Perspective on Industrial Waste Incineration (Published December 1992)

No. 50  Estimating Environmental Concentrations of Chemicals using Fate and Exposure Models (Published November 1992)

No. 51  Environmental Hazard Assessment of Substances (Published January 1993)

No. 52  Styrene Toxicology Investigation on the Potential for Carcinogenicity (Published August 1992)

No. 53  DHTDMAC: Aquatic and Terrestrial Hazard Assessment (CAS No. 61789-80-8) (Published February 1993)

No. 54  Assessment of the Biodegradation of Chemicals in the Marine Environment (Published August 1993)

No. 55  Pulmonary Toxicity of Polyalkylene Glycols (Published December 1997)

No. 56  Aquatic Toxicity Data Evaluation (Published December 1993)

No. 57  Polypropylene Production and Colorectal Cancer (Published February 1994)

No. 58  Assessment of Non-Occupational Exposure to Chemicals (Published May 1994)

No. 59  Testing for Worker Protection (Published April 1994)

No. 60  Trichloroethene: Assessment of Human Carcinogenic Hazard (Published May 1994)

No. 61  Environmental Exposure Assessment (Published September 1994)

No. 62  Ammonia Emissions to Air in Western Europe (Published July 1994)

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No. 64  The Toxicology of Glycol Ethers and its Relevance to Man (Published August 1995)

No. 65  Formaldehyde and Human Cancer Risks (Published May 1995)

No. 66  Skin Irritation and Corrosion: Reference Chemicals Data Bank (Published March 1995)

No. 67  The Role of Bioaccumulation in Environmental Risk Assessment: The Aquatic Environment and Related Food Webs (Published October 1995)

No. 68  Assessment Factors in Human Health Risk Assessment (updated by TR No. 86) (Published August 1995)

No. 69  Toxicology of Man-Made Organic Fibres (Published April 1996)

No. 70  Chronic Neurotoxicity of Solvents (Published February 1996)
Toxicological Modes of Action: Relevance for Human Risk Assessment

No. 71 Inventory of Critical Reviews on Chemicals (Only available to ECETOC members) (Published August 1996)
No. 72 Methyl tert-Butyl Ether (MTBE) Health Risk Characterisation (Published June 1997)
No. 73 The Value of Aquatic Model Ecosystem Studies in Ecotoxicology (Published December 1997)
No. 74 QSARs in the Assessment of the Environmental Fate and Effects of Chemicals (Published June 1998)
No. 75 Organophosphorus Pesticides and Long-term Effects on the Nervous System (Published December 1998)
No. 76 Monitoring and Modelling of Industrial Organic Chemicals, with Particular Reference to Aquatic Risk Assessment (Published January 1999)
No. 77 Skin and Respiratory Sensitisers: Reference Chemicals Data Bank (Published August 1999)
No. 78 Skin Sensitisation Testing: Methodological Considerations (Published December 1999)
No. 79 Exposure Factors Sourcebook for European Populations (with Focus on UK Data) (Published June 2001)
No. 80 Aquatic Toxicity of Mixtures (Published July 2001)
No. 81 Human Acute Intoxication from Monochloroacetic Acid: Proposals for Therapy (Published November 2001)
No. 82 Risk Assessment in Marine Environments (Published December 2001)
No. 84 Scientific Principles for Soil Hazard Assessment of Substances (Published July 2002)
No. 85 Recognition of, and Differentiation between, Adverse and Non-adverse Effects in Toxicology Studies (Published December 2002)
No. 86 Derivation of Assessment Factors for Human Health Risk Assessment (Published February 2003)
No. 87 Contact Sensitisation: Classification According to Potency (Published April 2003)
No. 88 Environmental Risk Assessment of Difficult Substances (Published June 2003)
No. 89 (Q)SARS: Evaluation of the Commercially Available Software for Human Health and Environmental Endpoints with Respect to Chemical Management Applications (Published September 2003)
No. 90 Persistence of Chemicals in the Environment (Published October 2003)
No. 91 Aquatic Hazard Assessment II (Published November 2003)
No. 92 Soil and Sediment Risk Assessment (Published December 2004)
No. 93 Targeted Risk Assessment (Published December 2004)
No. 94 Whole Effluent Assessment (Published December 2004)
No. 95 The Toxicology of Glycol Ethers and its Relevance to Man (Fourth Edition) Volume I and Volume II Substance Profiles (Published February 2005)
No. 96 Trends in Children’s Health and the Role of Chemicals: State of the Science Review (Published June 2005)
No. 97 Alternative Testing Approaches in Environmental Safety Assessment (Published December 2005)
No. 98 Risk Assessment of PBT Chemicals (Published December 2005)

Joint Assessment of Commodity Chemicals (JACC) Reports

No. Title
No. 1 Melamine (Published February 1983)
No. 2 1,4-Dioxane (Published February 1983)
No. 3 Methyl Ethyl Ketone (Published February 1983)
No. 4 Methylene Chloride (Published January 1984)
No. 5 Vinylidene Chloride (Published August 1985)
No. 6 Xylenes (Published June 1986)
No. 7 Ethylbenzene (Published August 1986)
No. 8 Methyl Isobutyl Ketone (Published May 1987)
<table>
<thead>
<tr>
<th>No.</th>
<th>Substance</th>
<th>Published/Published Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>Chlorodifluoromethane</td>
<td>October 1989</td>
</tr>
<tr>
<td>10</td>
<td>Isophorone</td>
<td>September 1989</td>
</tr>
<tr>
<td>11</td>
<td>1,2-Dichloro-1,1-difluoroethane (HFA-132b)</td>
<td>May 1990</td>
</tr>
<tr>
<td>12</td>
<td>1-Chloro-1,2,2,2-tetrafluoroethane (HFA-124)</td>
<td>May 1990 and updated by JACC No. 25 and No. 46</td>
</tr>
<tr>
<td>13</td>
<td>1,1-Dichloro-2,2,2-trifluoroethane (HFA-123)</td>
<td>May 1990 and updated by JACC No. 33</td>
</tr>
<tr>
<td>14</td>
<td>1-Chloro-2,2,2-trifluoroethane (HFA-133a)</td>
<td>August 1990</td>
</tr>
<tr>
<td>15</td>
<td>1-Fluoro 1,1-dichloroethane (HFA-141b)</td>
<td>August 1990 and updated by JACC No. 29</td>
</tr>
<tr>
<td>16</td>
<td>Dichlorofluoromethane (HCFC-21)</td>
<td>August 1990</td>
</tr>
<tr>
<td>17</td>
<td>1-Chloro-1,1-difluoroethane (HFA-142b)</td>
<td>August 1990</td>
</tr>
<tr>
<td>18</td>
<td>Vinyl Acetate</td>
<td>February 1991</td>
</tr>
<tr>
<td>19</td>
<td>Dicyclopentadiene (CAS: 77-73-6)</td>
<td>July 1991</td>
</tr>
<tr>
<td>20</td>
<td>Tris-/Bis-/Mono-(2 ethylhexyl) phosphate</td>
<td>May 1992</td>
</tr>
<tr>
<td>21</td>
<td>Tris-(2-butoxyethyl)-phosphate (CAS:78-51-3)</td>
<td>March 1992</td>
</tr>
<tr>
<td>22</td>
<td>Hydrogen Peroxide (CAS: 7722-84-1)</td>
<td>January 1993</td>
</tr>
<tr>
<td>23</td>
<td>Polycarboxylate Polymers as Used in Detergents</td>
<td>November 1993</td>
</tr>
<tr>
<td>24</td>
<td>Pentfluoroethane (HFC-125)</td>
<td>May 1994</td>
</tr>
<tr>
<td>25</td>
<td>1-Chloro-1,2,2,2-tetrafluoroethane (HFC-124)</td>
<td>August 1994 and updated by JACC 46</td>
</tr>
<tr>
<td>26</td>
<td>Linear Polymethylsiloxanes (CAS No. 63148-62-9)</td>
<td>September 1994</td>
</tr>
<tr>
<td>27</td>
<td>n-Butyl Acrylate (CAS No. 141-32-2)</td>
<td>August 1994</td>
</tr>
<tr>
<td>28</td>
<td>Ethyl Acrylate (CAS No. 140-88-5)</td>
<td>September 1994</td>
</tr>
<tr>
<td>29</td>
<td>1,1-Dichloro-1-fluoroethane (HCFC-141b)</td>
<td>December 1994</td>
</tr>
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<td>30</td>
<td>Methyl Methacrylate (CAS No. 80-62-6)</td>
<td>February 1995 and updated by JACC 50</td>
</tr>
<tr>
<td>31</td>
<td>1,1,1,2-Tetrafluoroethane (HFC-134a)</td>
<td>August 1995</td>
</tr>
<tr>
<td>32</td>
<td>Difluoroethane (HFC-32)</td>
<td>May 1995</td>
</tr>
<tr>
<td>33</td>
<td>1,1-Dichloro-2,2,2-trifluoroethane (HCFC-123)</td>
<td>February 1996 and updated by JACC 47</td>
</tr>
<tr>
<td>34</td>
<td>Acrylic Acid (CAS No. 79-10-7)</td>
<td>September 1995</td>
</tr>
<tr>
<td>35</td>
<td>Methacrylic Acid (CAS No. 79-41-4)</td>
<td>May 1996</td>
</tr>
<tr>
<td>36</td>
<td>n-Butyl Methacrylate; Isobutyl Methacrylate (CAS No. 97-88-1) (CAS No. 97-86-9)</td>
<td>December 1996 and updated by JACC 25</td>
</tr>
<tr>
<td>37</td>
<td>Methyl Acrylate (CAS No. 96-33-3)</td>
<td>September 1998</td>
</tr>
<tr>
<td>38</td>
<td>Monochloroacetic Acid (CAS No. 79-11-8) and its Sodium Salt (CAS No. 3926-62-3)</td>
<td>June 1999</td>
</tr>
<tr>
<td>39</td>
<td>Tetrachloroethylene (CAS No. 127-18-4)</td>
<td>December 1999</td>
</tr>
<tr>
<td>40</td>
<td>Peracetic Acid (CAS No. 79-21-0) and its Equilibrium Solutions</td>
<td>January 2001</td>
</tr>
<tr>
<td>41</td>
<td>n-Butanol (CAS No. 71-36-3)</td>
<td>March 2004</td>
</tr>
<tr>
<td>42</td>
<td>Tetrafluoroethylene (CAS No. 71-14-3)</td>
<td>December 2003</td>
</tr>
<tr>
<td>43</td>
<td>sec-Butanol (CAS No. 78-92-2)</td>
<td>March 2004</td>
</tr>
<tr>
<td>44</td>
<td>1, 1, 1, 3, 3-Pentafluoropropane (HFC-245fa)</td>
<td>June 2004</td>
</tr>
<tr>
<td>45</td>
<td>1, 1-Difluoroethane (HFC-152a)</td>
<td>September 2004</td>
</tr>
<tr>
<td>46</td>
<td>1-Chloro-1,2,2,2-tetrafluoroethane (HFC-124)</td>
<td>November 2004</td>
</tr>
<tr>
<td>47</td>
<td>1,1-Dichloro-2,2,2-trifluoroethane (HFC-123)</td>
<td>May 2005</td>
</tr>
<tr>
<td>48</td>
<td>Hexafluoropropylene (HFP)</td>
<td>September 2005</td>
</tr>
<tr>
<td>49</td>
<td>Vinylidene Fluoride CAS No. 75-38-7</td>
<td>November 2005</td>
</tr>
</tbody>
</table>
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<table>
<thead>
<tr>
<th>No.</th>
<th>Title</th>
<th>Published Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. 8</td>
<td>HAZCHEM; A Mathematical Model for Use in Risk Assessment of Substances</td>
<td>October 1994</td>
</tr>
<tr>
<td>No. 9</td>
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</tr>
<tr>
<td>No. 10</td>
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</tr>
<tr>
<td>No. 11</td>
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</tr>
<tr>
<td>No. 13</td>
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<td>August 1997</td>
</tr>
<tr>
<td>No. 14</td>
<td>n-Butyl Methacrylate and Isobutyl Methacrylate OEL Criteria Document</td>
<td>May 1998</td>
</tr>
<tr>
<td>No. 15</td>
<td>Examination of a Proposed Skin Notation Strategy</td>
<td>September 1998</td>
</tr>
<tr>
<td>No. 16</td>
<td>GREAT-ER User Manual</td>
<td>March 1999</td>
</tr>
<tr>
<td>No. 17</td>
<td>Risk Assessment Report for Existing Substances Methyl tertiary-Butyl Ether</td>
<td>December 2003</td>
</tr>
</tbody>
</table>
### Documents

<table>
<thead>
<tr>
<th>No.</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. 32</td>
<td>Environmental Oestrogens: Male Reproduction and Reproductive Development (Published January 1996)</td>
</tr>
<tr>
<td>No. 33</td>
<td>Environmental Oestrogens: A Compendium of Test Methods (Published July 1996)</td>
</tr>
<tr>
<td>No. 34</td>
<td>The Challenge Posed by Endocrine-disrupting Chemicals (Published February 1996)</td>
</tr>
<tr>
<td>No. 35</td>
<td>Exposure Assessment in the Context of the EU Technical Guidance Documents on Risk Assessment of Substances (Published May 1997)</td>
</tr>
<tr>
<td>No. 37</td>
<td>EC Classification of Eye Irritancy (Published December 1997)</td>
</tr>
<tr>
<td>No. 38</td>
<td>Wildlife and Endocrine Disrupters: Requirements for Hazard Identification (Published January 1998)</td>
</tr>
<tr>
<td>No. 39</td>
<td>Screening and Testing Methods for Ecotoxicological Effects of Potential Endocrine Disrupters: Response to the EDSTAC Recommendations and a Proposed Alternative Approach (Published January 1999)</td>
</tr>
<tr>
<td>No. 40</td>
<td>Comments on Recommendation from Scientific Committee on Occupational Exposure Limits for 1,3-Butadiene (Published October 2000)</td>
</tr>
<tr>
<td>No. 41</td>
<td>Persistent Organic Pollutants (POPs) Response to UNEP/INC/CEG-I Annex 1 (Published January 2000)</td>
</tr>
<tr>
<td>No. 42</td>
<td>Genomics, Transcript Profiling, Proteomics and Metabonomics (GTPM). An Introduction (Published April 2001)</td>
</tr>
<tr>
<td>No. 43</td>
<td>Contact Sensitisation: Classification According to Potency. A Commentary (Published July 2003)</td>
</tr>
<tr>
<td>No. 44</td>
<td>Guidance for the Interpretation of Biomonitoring Data (Published November 2005)</td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th>No.</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. 1</td>
<td>Workshop on Availability, Interpretation and Use of Environmental Monitoring Data 20-21 March 2003, Brussels (Published December 2003)</td>
</tr>
<tr>
<td>No. 2</td>
<td>Strategy Report on Challenges, Opportunities and Research needs arising from the Definition, Assessment and Management of Ecological Quality Status as required by the EU Water Framework Directive based on the workshop EQS and WFD versus PNEC and REACH - are they doing the job? 27-28 November 2003, Budapest (Published March 2004)</td>
</tr>
<tr>
<td>No. 3</td>
<td>Workshop on the Use of Human Data in Risk Assessment 23-24 February 2004, Cardiff (Published November 2004)</td>
</tr>
<tr>
<td>No. 4</td>
<td>Influence of Maternal Toxicity in Studies on Developmental Toxicity 2 March 2004, Berlin (Published October 2004)</td>
</tr>
<tr>
<td>No. 5</td>
<td>Workshop on Alternative Testing Approaches in Environmental Risk Assessment 7-9 July 2004, Paris (Published December 2004)</td>
</tr>
<tr>
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</tr>
</tbody>
</table>
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