Toxicity testing:
a more efficient approach

Gezondheidsraad **Voorzitter**
Health Council of the Netherlands

Aan de Minister van Volkshuisvesting, Ruimtelijke Ordening en Milieubeheer

Onderwerp : Aanbieding advies over een gerichtere beoordeling van stoffen
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Mijnheer de minister,

Op 29 oktober jl. ontving u van mij een brief over het concept-voorstel 'Criteria en beslisregels', waarin elementen uit de nota 'Strategisch Omgaan met Stoffen' (SOMS) nader zijn uitgewerkt (kenmerk U 2171/ES/mj/Algemeen). Ik liet u toen weten dat de Gezondheidsraad de laatste hand legde aan het advies 'Onderzoek gezondheidsrisico's stoffen: een gerichtere benadering'. Dat advies bied ik u hierbij aan. Het is op mijn verzoek opgesteld door een daartoe door mij geformeerde commissie van de Gezondheidsraad en beoordeeld door de Beraadsgroep Gezondheid en Omgeving. Tevens heb ik het advies voor commentaar voorgelegd aan de Commissie WGD van de Raad die advieswaarden voor beroepsmatige blootstelling aan stoffen afleidt. Ik heb dit advies vandaag ook aangeboden aan de Minister van Volksgezondheid, Welzijn en Sport en aan de Staatssecretaris van Sociale Zaken en Werkgelegenheid.

Zoals ik u al schreef biedt het zojuist bedoelde advies handreikingen voor de diverse fasen uit de nota SOMS. Net als in de nota staat in het advies het strategisch omgaan met stoffen centraal. De commissie die het advies heeft opgesteld, breekt een lans voor maatwerk bij het onderzoek naar de toxiciteit van stoffen. Zelf omschrijft zij haar oriëntatie zo: met welk doel wil men inzicht in die toxiciteit krijgen en welke koers kan men dan het best varen? De constructie van toxiciteitsprofielen kan naar de mening van de commissie vaak gerichter verlopen dan nu volgens diverse (internationale) bepalingen en richtlijnen is toegestaan. Groeiend inzicht in mechanismen van toxiciteit en nieuwe of in aantocht zijnde analysetechnieken maken dat steeds beter mogelijk.

Ik deel de visie van de commissie dat de bedoelde strategische flexibiliteit het niet kan stellen zonder een protocollering of standaardisatie op onderdelen. Daartoe moet het toxicologisch onderzoek voldoende worden gestimuleerd en gericht op de ontwikkeling van op het werkingsmechanisme georiënteerde methodieken. Verder dienen de regelgevende instanties ervoor

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te zorgen dat zulke nieuwe inzichten en technieken via doelmatige harmonisatieprocedures snel hun weg vinden naar de beoordelingspraktijk. Zonder het een en het ander loopt de strategische heroriëntatie mogelijk spaak.

Hoogachtend,

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Toxicity testing:
a more efficient approach

to:

the Minister of Housing, Spatial Planning and the Environment

the Minister of Health, Welfare and Sport

the State Secretary of Social Affairs and Employment

No. 2001/24E, The Hague, 20 November 2001

The Health Council of the Netherlands, established in 1902, is an independent scientific advisory body. Its remit is “to advise the government and Parliament on the current level of knowledge with respect to public health issues...” (Section 21, Health Act).

The Health Council receives most requests for advice from the Ministers of Health, Welfare & Sport, Housing, Spatial Planning & the Environment, Social Affairs & Employment, and Agriculture, Nature Preservation & Fisheries. The Council can publish advisory reports on its own initiative. It usually does this in order to ask attention for developments or trends that are thought to be relevant to government policy.

Most Health Council reports are prepared by multidisciplinary committees of Dutch or, sometimes, foreign experts, appointed in a personal capacity. The reports are available to the public.

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Contents

Executive summary

General recommendations on the principles and procedures for assessing the toxicity of chemicals entitled ‘Toxicology-based recommended exposure limits’ were published by a Health Council Committee in 1996. The present report, drawn up by the same Committee, elaborates the programme outlined then. It concentrates on the construction of an integral toxicity profile, including the relations between various types of toxicity research.

The Committee notes that, over the years, an international system of guidelines and assessment principles has come into being and proved its worth in practice. At the same time, however, the Committee points out that there are problems and limitations: the degree of focus on new toxicological ideas is lagging behind, and procedures are slow and sometimes rigid. The Committee urges a strategic approach to generating and interpreting toxicological data. More allowance needs to be made for the mechanisms by which chemicals work, and how and to what extent people are – or can be – exposed to them. Chapter 2 describes current developments in toxicology. Chapter 3 illustrates this with more detailed considerations of four end points, viz. acute toxicity, skin sensitisation, neurotoxicity and carcinogenicity. Both chapters focus on the mechanistic point of view. The Committee believes it is essential to understand how chemicals work, if their toxicity – or potential toxicity – is to be properly assessed. Understanding of this has grown substantially in recent years as a result of new or improved analytical techniques. The Committee reviews a selection of methods. SARs (structure-activity relationships) express relationships between the physico-chemical properties of a substance and its biological – or toxic – activity. Biokinetic research is

concerned with the intake, distribution, metabolism and elimination of particular chemicals. Toxicodynamic analysis looks at the interaction between molecules of the chemical in question (or its metabolites) and biologically important molecules such as DNA and cellular receptors. So called 'high throughput' systems, automated procedures to screen the potential toxicity of large numbers of chemicals, are also emerging. In vitro and in vivo techniques can complement each another in this mechanistically oriented research.

The rapid advances being made in genetics are also bringing a new set of analytical tools within reach. This includes transgenic animal models and, perhaps in the slightly longer term, toxicogenomics, research into changes in gene expression caused by toxic chemicals. The Committee suggests that particular attention be paid to this latter subject.

The four end points selected as examples display wide variation when it comes to knowledge of mechanisms and the scope for applying new analytical techniques. Hitherto, such techniques have proved most effective in the case of genotoxic carcinogenicity, but the Committee would like to look ahead as well. Its interests are not confined to existing technology, it is also focusing on developments that are at an advanced stage or could become possible in the near future. Validation of methods is a constant concern here. The Committee recommends that validation be facilitated and promoted on an international basis.

As a result of its deliberations, the Committee urges a strategic approach to the orderly collection and interpretation of toxicological data. Particular emphasis should be given to the host of chemicals about which little, if anything, is known. The Committee presents a flow chart showing various methods with associated levels of safety evaluation. What level is necessary or adequate depends not only on the intrinsic toxicity of the chemical in question but also on policy considerations.

There are two key principles here. First, the Committee considers that simple analytical techniques concerned with deeper levels of biological organization should be used before more specialized, increasingly complex methods. In practice this means starting with SAR analysis, if possible with 'high-throughput' procedures, and in vitro experiments. Based on these results, and depending on the circumstances, in vivo tests can then be done.

Secondly, the way the chemical is used and the associated exposure profile need to be borne in mind. In the case of the former there are considerations e.g. of utility versus risk. In the case of the latter the Committee urges application of the TTC (*Threshold of Toxicological Concern*), which operationalizes the idea that certain exposure levels are insignificant from the toxicological point of view. Work still needs to be done, however, on various aspects of this operationalization, in the Committee's

opinion. If the expected intake of a chemical is below the TTC, the authorities could decide not to have any more safety evaluations carried out, or to give them very low priority. If exposure is toxicologically relevant, the exposure analysis will, as a rule, become more detailed with increasing levels of toxicological analysis. In other words, the authorities will want to know more and more precisely who are – or could be – exposed and the expected extent of the exposure.

The flow chart is a pragmatic guide, not a binding protocol. In the Committee's opinion, it is up to the experts to decide how best to apply this general system in specific contexts and to specific chemicals. As already indicated, validation of methods is one of the points to be taken into consideration here. In addition, the binary (yes/no) decision-making rules are not absolute. A 'no', for instance, is not necessarily the final word. It should instead be taken to mean that more in-depth evaluation has low priority, at least for the time being. However, the Committee does take the view that various modules and decision-making rules should be protocolled and harmonized. Far from seeing flexibility and standardization as incompatible, the Committee takes the view that they represent a duality.

Chapter 1

Introduction

In recent decades, understanding of the cellular or molecular action of chemicals has grown steadily. However, regulatory authorities and toxicologists have been slow to review their often time-consuming procedures for assessing the toxicity of such substances. There is still a very long list of substances about which little or nothing is known. This report explores the possibility that there may now be better ways of addressing these intractable problems.

1.1 Current assessment system

Under various international provisions and guidelines, a full human toxicological assessment of substances comprises four stages: (1) establishment of the toxic properties, (2) determination of the link between exposure and toxic effect, (3) determination of exposure, and (4) characterization of the health risk. Stage (1), often termed '*hazard identification*', and stage (2) together form a toxicity assessment in the strict sense of the term. Stage (4), which is based on a combination of the previous three stages, is known by the generally synonymous expressions '*risk assessment*' and '*safety evaluation*'.

In the absence of sound epidemiological data, a toxicity assessment under the system referred to above is based chiefly on the findings of routine research using experimental animals (screening for mutagenicity and carcinogenicity via validated in vitro procedures are exceptions to the rule). Bodies such as the EU (European Union) and the OECD (Organization for Economic Co-operation and Development) have

drawn up guidelines for the design and performance of such experimental animal research. Moreover, these guidelines have been updated at reasonably regular intervals (CEC93a, CEC93b, CEC96, Lee96). The resultant harmonisation of toxicity studies has contributed considerably to the quality and comparability of the data generated in various laboratories.

Furthermore, the information required and the studies to be performed depend on the production volume and application of a substance (CEC93a, CEC93b, CEC96). Accordingly, the European Commission has recently issued a proposal for bringing new and existing substances within a single assessment regime, which also takes (possible) exposure into account (CEC01).

Information on the biokinetics (absorption, distribution, metabolism and excretion) of substances may, within this assessment framework, help in interpreting the experimental animal data in question. This information thus has a mainly supplementary function. In this way, it is possible to identify, for instance, any differences between experimental animals and humans. Guidelines are on the drawing board in this area as well, some of which concern the usability of in vitro methods (CEC92; ECE92).

The corpus of data thus generated (or already available) forms the basis for decision-making where the protection of public health is concerned. This may entail a classification on the basis of toxic properties, often with a weight of evidence (some examples are carcinogenicity, reproductive toxicity and corrosiveness). Another procedure, for which more data is generally needed, is the derivation of toxicology-based recommended exposure limits (GR96). As stated, this system of guidelines and assessment principles has proven its worth in practice. However, objections and limitations can also be cited. The most important of these are reviewed in the following sections.

1.2 New voices

Within scientific circles, the call for a review of the current assessment system has been growing ever louder. Toxicologists engaged in fundamental research argue that data on the mechanisms of action of substances in combination with model-related considerations should be given a more prominent place. They see potential in all manner of new analytical techniques. These include structure-activity relationships, whereby harmful effects of as yet uninvestigated substances can be predicted. There are also '*high throughput*' systems, which give an impression of the hazardous properties of a vast array of substances. Other techniques include methods for selecting the type of experimental animal and effect that are most relevant for humans, where there are good grounds for further toxicological research. Finally, there are methods

for extrapolation from high to low exposure levels, methods for extrapolation from experimental animals to humans, and methods for charting differences in sensitivity between individuals (GR98).

The chemicals industry in Europe, North America and Japan has also undertaken activities in this area, as illustrated by its so-called '*Long-range Research Initiative (LRI)*'. This LRI incorporates a financial injection to fundamental research on carcinogenicity, neurotoxicity, immunotoxicity and allergy, respiratory toxicity and disruption of hormonal processes (CII98, CII00, ECE99). The purpose being to develop more sensitive methods for measuring effects and to facilitate more efficient toxicity and risk assessments. This strategic approach is thought to be desirable because the application of substances – and thus the exposure profile (who are exposed, and to what extent?) – exhibits a high degree of variation. With a view to achieving efficient health protection, it would therefore be preferable not to test all substances in an equally detailed way or in a uniform fashion. As mentioned, production volume is a factor, but the thinking is that many more steps can be taken towards a tailor-made approach. In other words, many are advocating a strategic approach and claiming that new analytical techniques may then be of use.

1.3 Committee, terms of reference and procedure

A general advisory report on the principles and procedures for toxicity and risk assessments entitled 'Toxicology-based recommended exposure limits' was published by the Health Council of the Netherlands in 1996 (GR96). At that time, the Committee (whose current composition is set out in Annex B) which drew up that programme-based advisory report at the request of the President of the Health Council of the Netherlands stated that it would devote more attention to the above-mentioned plea for a change in direction in research on the toxicity of substances (see Annex A). This advisory report contains the results of its deliberations.

Before setting out its approach, the Committee would first like to sketch out the socio-political background against which scientific developments take place. The strong emphasis on protocol-based animal testing is an increasing focus of criticism, much of which originates from society in general. There are three main objections. Firstly, many people are urging the reduced use of experimental animals on ethical grounds. Secondly, there are increasingly frequent signals from policymakers and governmental bodies that the assessment procedures for substances are too slow. In addition, nothing at all is known about the toxicity of a whole host of compounds. This stagnation can, to some extent, be attributed to legal and administrative factors. Another major factor is the time-consuming nature of protocol-based animal testing, which entails mandatory comprehensive schemes. Thirdly, these extensive studies

involve considerable expense. Hence the growing interest in faster assessment procedures based on new techniques that do without animal testing. Recently, the '*Strategy memorandum on dealing with substances*' (SOMS) appeared in this context (VROM01). In this memorandum, the government sets out how a more efficient policy on substances can be brought about. Thus, socio-political and scientific developments are more or less in step with one another.

The Committee takes new initiatives for the stepped and targeted gathering – and interpretation – of toxicological information as the point of reference for its advisory report. It has a 'strategic toxicity profile' in mind. Without wanting to go into too much detail here, it makes an attempt to concentrate thinking by outlining a number of principles for the formulation of such profiles. The first important guiding principle is the application of the substance under consideration, and the associated exposure profile. Even now, toxicological assessments must draw a distinction between industrial chemicals, pharmaceutical products, pesticides and food additives, for example. Further differentiation is possible, based on the groups of people that are (potentially) exposed to the substance. It is also possible to draw a distinction using the way in which such exposure takes place and the route involved. This approach provides a sounder basis for distinguishing between more and less suitable types of research.

A second key principle relates to the Committee's initial topic for examination, namely early consideration of data on the substance's mechanism of action. Such information may sometimes be sufficient for certain policy initiatives, for example classification in a hazard class. In many cases, it can also serve as a guide for further research. For the record, the Committee does not wish to imply that protocol-based toxicological research must *per se* make way for mechanistic (in vitro and in vivo) research or for model-based approaches. Each of the various analytical techniques has its own value and limitations. Each also derives its evidential value in part from its linkage with the other information sources. In the Committee's view, it is thus mainly a question of articulating in greater detail the way in which the building blocks required for a specific objective can be selected and combined. Not only do new elements need to be incorporated, but existing tools must also be reassessed. In other words, what is the purpose of seeking to understand the toxicity of a substance and what is the best way of achieving this?

Of course, with such a general subject as toxicity profiles, the question immediately arises as to the scope, definition and focus of the advisory report. The Committee's objective is limited and clearly defined: to outline the main features of new toxicological developments on the basis of which the Dutch government can further shape its policy described in the 'SOMS' memorandum. The Committee has therefore decided to highlight the prospects of new methods based on toxic

mechanisms of action. However, merely by way of illustration, a number of end points are also given special attention. Where possible, the Committee indicates what has been validated and is thus ready for use. It also identifies those that have not as yet fully matured and those that are at an early stage of development. It provides details of the difficulties that are still to be overcome and what action can be taken to facilitate this. Whether the implementation of new methods is actually appropriate and, if so, under what conditions, also depends on factors outside the scientific arena, such as efficiency.

The Committee's human toxicology perspective entails a further definition of the terms of reference. Firstly, epidemiological information and research techniques are not examined separately, although they are mentioned a number of times in the advisory report. And secondly, ecotoxicological elements of the assessment system are ignored.

The format of the advisory report is as follows. Chapter 2 contains a general survey of new developments in toxicology. Chapter 3 incorporates an outline of the various forms of stepped toxicity profile structures (based on the literature relating to four end points: acute toxicity, dermal sensitisation, neurotoxicity and carcinogenicity). Chapter 4 is devoted to the general assessment strategy advocated by the Committee. The latter elucidates its ideas with the aid of two decision-making flow charts. As mentioned above, the emphasis here is on the targeted construction of toxicity profiles, which is pre-eminently important for new or poorly researched substances. After all, existing substances with a high production volume are often well understood. While flow charts of this kind will not then be required, the available data will have to be fully assessed.

Chapter **2**

New developments in toxicology

In its 1996 programme-based advisory report, the Committee gave an overall explanation of the building blocks underlying a toxicity profile (a selection of in vitro and in vivo data) and of techniques for making links between these elements (such as biokinetic and toxicodynamic models). However, further details were generally lacking regarding the way in which such elements can be combined more effectively with a view to toxicity and risk assessments. This advisory report contains further proposals. Before setting out its ideas, the Committee feels that it would be useful to explain current developments in toxicology in somewhat greater detail.

2.1 The mechanistic perspective

In toxicology, as in other areas of biomedical research, ever better methods are being developed to elucidate the mechanisms by which damage to health occurs. Researchers are gaining an ever clearer understanding of the molecular and cell biological processes that underlie observed health effects in humans and experimental animals (GR98). The first links in cause-and-effect chains of this kind relate to what is known as biokinetics: the absorption, distribution, metabolism and excretion of substances. There is a desire to know what fraction of the substance under investigation or its reactive metabolites (conversion products) reaches certain organs and can start to interact there with biologically important macromolecules, or simply biomolecules. This fraction is known as the biologically available, biologically effective or target dose. In current biokinetic research, there is considerable focus on the identification of

toxic metabolites. Another major area of interest is the role of enzymes (such as cytochrome P450 (CYP), epoxy hydrolase and glutathione transferase) in the formation and removal of these metabolites. By conducting such (in vitro and in vivo) research on various species, an understanding can be gained of any differences between experimental animals and humans (ECE92). Possible biokinetic differences between high and low exposure can also be traced in this way. Both types of understanding can provide a basis for deriving toxicology-based recommended exposure limits, in relation to which various extrapolation problems generally emerge.

A third field of biokinetic research concerns hereditary variation in humans. Thus, differences in the activity of certain enzymes (a phenomenon known as genetic polymorphism) are connected with the rate at which toxic metabolites are formed and substances or their metabolites are eliminated (Bol01, Wol00). Research on such differences may help in better assessing the risk run by certain individuals or groups (Wel92). A promising in vivo technique in this context is the application of so-called 'knockout' animal models. In these models, certain enzymes, e.g. CYP2E1, are rendered ineffective by genetic modification. CYP2EI is involved in the metabolism of a number of important industrial chemicals, such as benzene, ethylene and 1,3-butadiene. The role of this enzyme can be studied by comparing biotransformation in normal and modified experimental animals (Gon98, Jac99).

Toxicodynamics involves the interaction between molecules of the substance under consideration (or its metabolites) and biomolecules such as DNA, activating and deactivating receptors. It also addresses the further consequences of such interaction, such as the ways in which manifest health effects are produced. One example of a toxicodynamic process is when a substance binds to DNA, leading to the induction of gene mutations. Another is when it binds to a hormone receptor, leading to the disruption of hormonal processes. Substances can also bind to acetylcholinesterase, leading to the disruption of nerve impulse transmission. As a rule, less is known about the toxicodynamic side of things than about biokinetic aspects. Chemical carcinogenesis is a favourable exception, particularly the genotoxic variant (in which interactions with DNA occur) (But92, Gre00, Loh99, Pre98, Wri95). The Committee deals with this aspect in more detail in Chapter 3. At this point it would like to discuss two other methods. At the present time, a great deal of work is being carried out on the development of transgenic animal models (in which hereditary material from one species is incorporated into another) in order to be able to assess the carcinogenicity of substances more quickly (Gre00, Ten99). The Committee feels that it will not be long before this method is more broadly applied in the field of toxicology. Techniques for studying changes in gene expression caused by toxic substances (often termed *toxicogenomics*) have also been the focus of increasing attention in recent years. The Committee makes a number of observations on this later in this chapter.

Data on biokinetics and toxicodynamics gains in evidential value when described in a mutual context. The most compact form of description consists of (mathematical) models. It can be gathered from the above that biokinetic modelling (or, more precisely, PBPK: *Physiologically Based Pharmacokinetic Modelling*) is the furthest advanced in relative terms (And94, Fed95, Fre93, Kri92, Lau00). The requisite data on aspects such as the distribution of a substance between tissues and blood, any conversion processes and binding to biomolecules can be gleaned from both in vitro and in vivo studies (ECE92). A brief outline of research techniques in current use and those still under development in this area is provided below. In vitro research, in which cellular fractions of experimental animals and humans (where available) are used, provides mainly qualitative information in this regard. The predictive value of biokinetic models (relating to the biological effective dose in certain organs) can be assessed by comparison with data obtained via animal experiments. Discrepancies may then justify further research, for example on the precise metabolism of a substance. PBPK models of proven value have been developed for substances such as styrene (Ram84), methylene chloride (And87), chloroform (Re90), methanol (Hor92), and 1,3-butadiene (Joh93, Med94). In the case of toxicodynamic modelling, the situation is less favourable. As mentioned, this is bound up with the generally deficient understanding of toxic mechanisms of action. Most models in this area describe the action of carcinogenic substances (ECE96, GR96a, GR98). Models for other end points are also starting to appear in the literature in dribs and drabs. One example is the effect on the physiological development of children (Fau99, Ler96).

What are known as '*high throughput*' systems (automated procedures whereby possible hazardous characteristics of large numbers of substances can be investigated) are also emerging.

The Committee wishes to conclude with an observation of a more practical nature. Biokinetic models in particular can be developed or refined for all manner of substances. In other words, this is possible if time and money are no object, or are of only secondary importance. Bodies engaged in the field of toxicity and risk assessments cannot, however, ignore such considerations.

2.2 Structure-activity relationships

Structure-activity relationships (SARs) are links between the physicochemical structure of a substance (chemical reactivity, for example) and its biological activity. Computerised expert systems have now been developed that enable the toxicity of certain substances to be predicted (Rid96, Sil96). Thus, fragments of molecules that react with proteins indicate their capacity for inducing dermal sensitisation. Another example concerns the search for '*structural alerts*' for interactions with DNA (Loh99).

To date, SARs have proved valuable in connection with genotoxic carcinogenicity, mutagenicity, skin and eye irritation and dermal sensitisation. So-called QSARs, which express structure-activity relationships in statistical terms, have been formulated for the same end points (Bar98a, Bar98b, Bas95, Sil96).

Nevertheless, predictions based on (Q)SARs are generally characterised by considerable uncertainty. Additional research on details of metabolic processes may then sometimes help. To take one example: according to in vitro research, the rate at which the biologically active epoxy groups of glycidyl ethers are inactivated displays a high degree of variation (Boo00). Thus their mutagenic potency is also expected to vary widely, something that would not be picked up by a simple SAR analysis. Validation is also key to this method. Validated (Q)SARs provide a valuable way of rapidly screening the toxic potency of substances, or of prioritising substances with a view to further toxicity research (EPA00).

2.3 In vitro methods

Toxicologists have a selection of in vitro techniques at their disposal. There are in vitro preparations and associated analytical methods for a large number of organs and tissues. These range from isolated organs to subcellular fractions, for both experimental animals and humans (Spi98). Research on the various forms of organ toxicity is now so far advanced that it can safely be said to encompass a number of specialist fields. The Committee can provide only an outline of the situation in this description and would refer the reader for further details to a number of summary articles (Cos98, Cur98, Gol98, Gui98, Kar98, Par98, Pfa98, Spi98, Spi98a, Zac98). However, a number of aspects are considered in further detail in Chapter 3.

Let us start with an observation that applies generally to all organs. The more the tissue structure remains intact, the less the difference from the in vivo situation and the greater the evidential value assumed by in vitro studies. There are practical objections to this, however. For example, work with organs (or parts thereof) calls for relatively extensive technical facilities. Furthermore, the number of organs available is often very limited (human organs are scarce). Cells, cell lines and cell components can usually be handled more easily and are more widely available. However, the activity of cellular fractions (for example, microsomal enzymes), and thus their usability, can decline rapidly. Some particularly interesting questions recur again and again in discussions of the numerous organ-specific in vitro tests. Many want to know how well the findings match in vivo data, whether there are any indications of interspecies variation, and whether validation studies have been carried out.

In vitro methods are especially well suited to initial screenings for toxicity. In general terms, these may be based on mechanisms of action or purely on correlations

with findings from in vivo studies. The former will generally be preferred since they provide a basis for explaining such correlations. This may entail determinations of molecular interactions between substances (or their active metabolites) with biomolecules and measurements of the consequences of such interactions (for example, gene expression). Earlier in this chapter, however, the Committee pointed out that the mechanism of toxicity for most end points is still largely unclear. Generally speaking, only a few stages in the process are known. The Committee therefore feels that, for the time being, the findings of in vitro research have only limited use as a basis for toxicity and risk assessments. Some exceptions to this are the tests for genotoxicity and eye and skin irritation, which have now been adequately developed and laid down in directives.

Currently, the findings of in vitro research mainly offer scope for the improved interpretation of in vivo data. Nonetheless, the Committee expects that developments in molecular and cell biology will increase the predictive value of such in vitro methods in the next few years. As a result, better guidance can be given to in vivo research. Secondly, there will be an ever better understanding of how best to integrate in vitro and in vivo data. This may result in better biokinetic and toxicodynamic models, for example. An important requirement in this regard is that tests be performed using both human and animal material. This will enable any interspecies differences to be identified and taken into account during further research and evaluations.

2.4 In vivo methods

The Committee pointed out in the introductory chapter that in vivo studies, particularly protocol-based animal studies, are at the heart of the current assessment system. As it explains in the following chapter, efforts by toxicologists are in part aimed at developing short-term screening methods with conventional experimental animals. The Committee considers the mechanistic perspective to be of pivotal significance in this regard. It considers three methods to be of particular importance in this context: the use of genetically modified animals, the application of biomarkers, and analysis on the basis of gene expression (‘*toxicogenomics*’).

In the case of genetically modified experimental animals, one or more genes are eliminated or, conversely, incorporated. To date, these animal models have chiefly been used to clarify issues involving mutagenesis and carcinogenesis. Initial studies seem to indicate that this approach can identify carcinogenic substances more quickly. Furthermore, fewer experimental animals are required and the experience is less onerous for those that are used (Ten99). More details of this are provided in Chapter 3.

So-called biomarkers relate to certain links in the exposure-effect chain. These concern forms of internal exposure and premature biochemical effects that precede

manifest health damage (GR98). Examples of this type include the concentration of a substance in blood, the concentration of metabolites in urine and the formation of adducts (covalent bonds) to DNA and proteins, such as haemoglobin. The quantity of haemoglobin adduct is, for example, a suitable biomarker for monitoring exposure to genotoxic carcinogens (ECE89). Biomarkers for effects are rarer. Here, too, genotoxicity stands out: well-known examples include gene mutations and chromosomal aberrations in peripheral lymphocytes (see Chapter 3). Such biomarkers are less developed or entirely absent for other forms of toxicity. The usability of effect markers stands or falls, of course, by their predictive power. A great deal of research has still to be conducted on this. Molecular epidemiology, which is gradually gaining ground, is set to make a vital contribution (ECE89, GR98, Sil94, Wel92).

Nor has toxicology remained untouched by the enormous progress being made in genetics. Many people nurture high hopes of developing techniques to reveal changes in gene expression. This would provide detailed information about all kinds of toxicity mechanisms (Cor99, Far99, Nuw99). One benefit would be a better understanding of individual differences in sensitivity to substances. Various methods are under development for the measurement of gene expression: '*DNA microarrays*', on which segments of so-called cDNA are located; and '*DNA chips*', which contain much shorter segments. It is also possible to determine changes in the expression of proteins ('*proteomics*'). While it is still too soon for such techniques to be regularly used in toxicity and risk assessments, the Committee anticipates that their development will progress rapidly. It recommends to devote a separate advisory report to this subject.

2.5 Biologically based assessments

The common thread in this chapter is that a toxicity or risk assessment can gain in precision or effectiveness if greater use is made of new methods, mostly along mechanistic lines. How such an assessment can be formulated for substances about which little or nothing is known is explained in greater detail in Chapter 4. However, the interpretation of currently available toxicological information can also benefit from these new methods. In its programme-based advisory report, the Committee has therefore explained how toxicology-based recommended exposure limits are derived in accordance with the current assessment system. This is done using animal testing data, and with the aid of extrapolation and safety factors (GR96). Every stage in this derivation process qualifies for further biological substantiation. The Committee briefly lists them again with reference to the foregoing sections and the examples provided therein.

Exposure-effect relationships (on the basis of animal experiments) can be determined more precisely if better indicators of exposure and effect are used. In vitro

and in vivo data on biokinetics and toxicodynamics, or biomarkers, can provide the necessary information in this context. The importance of this is that it will provide a better understanding of the profile of the exposure-effect curve in the low-dose range (is there likely to be a threshold?; how marked is the curve?). When setting up animal experiments, an appropriate choice of dosage can also generate more information on such curves. Furthermore, mechanistic data provides toxicologists with a clearer view of the comparability of humans and experimental animals and of individual differences in sensitivity. In other words, information on mechanisms of action can provide grounds for the selection of animal species for in vivo studies and of interspecies and intraspecies factors in the derivation of recommended limits.

Broad applicability is conditional on the validation of techniques. The Committee has found that some methods have still not been adequately developed. Others have, as things currently stand, proven their value for only a few end points. The following chapter contains a further illustration of these findings. However, the Committee has not restricted itself to the current state of knowledge. Methods that are currently in an embryonic state may become reliable tools in the future.

Chapter 3

Four examples

The previous chapter examined the main ways in which toxicology has developed in recent decades and the direction in which fundamental and applied research appears to be heading in the next few years. Here the Committee focuses in greater detail on the significance of these developments for toxicity and risk assessment. A more detailed and integrated examination of this kind cannot cover the entire field of toxicology. To illustrate the variation in scope for applications that exists with the current state of knowledge, the Committee takes a closer look at four end points, namely: acute toxicity, dermal sensitisation, neurotoxicity and carcinogenicity.

'Variation' and 'illustrate' are key words here, so far as the Committee is concerned. It is evident from the literature that a number of proposals have been made to achieve gradual collection of toxicity data. The various stages have been broken down into differing degrees of detail, depending on the level of understanding of the mechanisms underlying specific forms of toxicity. Following on from this, there are also differences in the appraisals made when passing from one phase to another. Thus, the various examples cannot simply be reduced to a single basic pattern. The Committee does not tackle this issue in the present chapter. It merely wishes to show that interest in the phased design of toxicity profiles is growing in line with mechanistic information. The examples thus provide a means of approaching the general examination in the following chapter. The main point emphasised by the Committee there also applies here. No strategic approach of this kind can succeed without access to expert opinion. The latter must determine how the assessment can best be carried out in specific situations and for individual end points .

3.1 Acute toxicity

This form of toxicity entails (non-local) harmful effects that occur immediately or shortly after exposure to a substance. While pathological research can identify the organs that sustain damage in this context, it is not yet standard procedure. Pathological research is expected to gain in significance with the introduction of new methods, in which sublethal doses are administered. Research on mechanisms of action is helping to explain how this acute damage comes about. One mechanism involves the binding of the substance in question (or its metabolites) to biomolecules such as DNA and certain proteins. The Committee briefly reviews established and new research methods below.

Mechanisms of action

The form of damage referred to here can result from the disruption of all manner of processes that influence the viability or functioning of cells. These include damage to the integrity of membranes, disruption of cell metabolism and inhibition of protein synthesis. Often, too little is known about exactly how such substances produce their harmful effect. Nevertheless, it is known that certain disturbances can arise in every cell type. Basic cytotoxicity is then said to be involved. On the other hand, it is possible that certain differentiated (i.e. organ-specific) cells display increased sensitivity to a substance (selective cytotoxicity). Given the multitude of possible disruption mechanisms, the findings of in vitro studies cannot be expected to match up closely with acute toxicity assays from animal experiments. This will be dealt with in more detail presently.

Tested methods

The best-known regulating in vivo assay is the LD50 (or LC50) test: the dose (expressed in mg/kg) or the inhaled concentration (expressed in mg/m3) administered orally or cutaneously to experimental animals that brings about death in 50% of cases. Protocols that have been formulated under the auspices of the OECD have been in existence for some time for the performance of such tests. The first guideline, dating from 1981, can no longer be used in Dutch laboratories (OECD81). This has gradually been replaced by in vivo procedures that involve the use of fewer experimental animals. Examples include the so-called limit test (OECD87), the '*fixed-dose*' procedure (OECD92), the '*toxic-class*' method (OECD96) and the '*up-and-down*' procedure (OECD00). For details, the Committee would refer the reader to the sources

cited. In parallel with this, toxicologists have developed in vitro methods for determining basic cytotoxicity (Sei96). However, these alternative methods have not yet been accepted and adopted in guidelines.

Mechanistically oriented methods

Toxicologists have not yet managed to develop QSARs that are capable of predicting the acute toxicity of substances for regulatory purposes. Some stumbling blocks here are the multitude of possible effects and the lack of knowledge regarding mechanisms of action.

The MEIC (*Multicentre Evaluation of In Vitro Toxicity*) project produced the most elaborate validation research on in vitro procedures for determining cytotoxicity. Thirty laboratories participated in this project (Cle96, Sei96, Wal98). The basic cytotoxicity of 50 reference substances was determined using 68 different methods. There was a close match between the findings of these in vitro procedures and those of associated in vivo studies on humans and experimental animals (Cle96). The cell type used and the exact end point in question mattered less. Researchers have explained these findings by postulating that most substances are able to disrupt critical processes that are important for all cell types. One validated method has been accepted by the EU for measuring basic cytotoxicity, this is the so-called NRU ('*Neutral Red Uptake'*) test (Spi94). However, this approach does not suffice where metabolically activated substances are concerned. The use of liver cells is then necessary. Such cases also require research to be conducted using other differentiated cells (nerve cells, for instance) owing to possible organ-specific effects (Wal98).

The more that is known about the biokinetics of a substance, the greater the predictive value of in vitro studies. After all, in vitro concentrations can be converted to in vivo concentrations via biokinetic models. This generally complex procedure is not practicable for all substances, however, so simpler approaches have been developed. With the aid of these methods, LD50 values for experimental animals can be derived within safe limits of precision from EC50 values obtained via in vitro research (Gul94).

Strategic approach

Participants in a meeting of the ECVAM (*European Center for the Validation of Alternative Methods*) have proposed a stepped test strategy for acute toxicity. Under this strategy, in vitro studies precede animal tests. The aim is twofold: firstly, classification (labelling) of substances, and secondly to gain an understanding of mechanisms of action (Sei94, Sei96). The Committee points out the ideal nature of the

proposal: it can be used if sufficiently validated methods are available. The strategy consists of the following phases:

Phase 1 The in vivo biokinetics (for example, possible skin penetration) are predicted on the basis of physicochemical information (where possible, in the form of QSARs) and biokinetic in vitro data.

Phase 2 Basic cytotoxicity is determined by means of an in vitro study. The findings (such as the EC50) are converted to an in vivo LD50 with the aid of data obtained in phase 1. Where there is high toxicity (to be determined by experts), further research on acute toxicity is unnecessary. Otherwise, one moves on to phase 3.

Phase 3 Tests with liver cells must show whether metabolic activation occurs. Determination of cytotoxicity in these cells takes place at the same time. If the substance turns out to be highly toxic, further research can be dispensed with. If not, phase 4 follows.

Phase 4 In vitro research is conducted on organ-specific cytotoxicity. The acute toxicity is classified on the basis of the lowest EC50 from phases 2, 3 and 4.

Phase 5 If the substance ends up in the lowest class (‘no label’), a limited animal experiment is considered in order to ensure that toxic potency is not underestimated.

Evaluation

Without doubt, the ECVAM vision offers a promising prospect. However, the Committee considers that the available in vitro methods do not as yet allow for sufficiently reliable prediction of the acute toxicity of substances in connection with human exposure. This is partly due to uncertainties associated with the conversion from in vitro to in vivo concentrations. In vivo methods cannot therefore be dispensed with for the time being. However, it is useful that information on the toxic potency of a substance can be obtained via in vitro methods. This information can be used for setting up animal experiments, for example for selecting the doses to be administered.

3.2 Dermal sensitisation

Dermal sensitisation is the result of an immunological reaction that occurs directly or after repeated exposure to an allergen. The delayed type of hypersensitivity occurs frequently in the general population and in workers in the chemicals industry (ECE90).

Mechanisms of action

'Delayed' hypersensitivity reactions generally involve small molecules known as haptens. First, a molecule of this kind penetrates the horny layer of the skin and then comes into contact with the epidermis. Here, the hapten (or its active metabolite) reacts with certain proteins in what are known as dendritic cells (including Langerhans' cells), forming adducts. This sets in train a series of reactions in the immune system. It is important in this context that so-called '*memory*' cells spread throughout the body via the lymph vessels. These cells have a receptor via which, on later exposure to the allergenic substance ('*challenge*'), they again undergo an interaction resulting in the production of cytokines and chemokines. In people sensitised in this way, this eventually leads to contact dermatitis (ECE00, Sil96). Both the level of induction of dermal sensitisation and the degree of expression of contact dermatitis depend on the dose (Kim97). It also seems as if allergenic substances have a threshold value, i.e. a level of exposure below which dermal sensitisation does not occur (Kim99).

Tested methods

The two methods most used are the '*guinea-pig maximisation test*' (GMPT) (Mag69) and Buehler's '*occluded patch test*' (Bue65). Both methods are based on subjective assessment of dermal reactions following '*challenge*'. The GMPT method is more sensitive and has therefore become the preferred choice of the regulatory authorities. The performance procedures are specified in standard protocols (OECD92a). Adjustments were recently proposed according to which fewer experimental animals are necessary (ECE00).

Mechanistically oriented methods

QSAR models have been developed on the basis of the reactivity of the substance (or its active metabolite) and its capacity to reach target cells in the epidermis (Bar95). As described in section 2.2, this involves the presence of reactive fragments in the molecule ('*structural alerts*'). These '*structural alerts*' can be identified via computerised expert systems, such as DEREK (*Deductive Estimation of Risk from Existing Knowledge*) (Bar94). A validation study of the predictive value of '*structural alerts*' for dermal sensitisation identified in this way shows that the system's sensitivity is good (2 false negatives out of a total of 135 sensitising substances), but that specificity is moderate (22 false positives out of a total of 120 non-sensitising substances). The latter is probably attributable to the low dermal penetration of the substances in question (Sil96).

Two new in vivo methods have become available over the last decade, namely the '*mouse ear swelling test*' (MEST) (Gad86, Sil96) and the '*local lymph node assay*' (LLNA) (Kim89, Sil96, ECE00). Both methods help reduce the number of experimental animals required. The OECD has proposed using the methods as a screening test (OECD92a). At the present time, the LLNA is preferred to the MEST: firstly, the end point can be positively determined with the former, while this test is also less onerous for experimental animals (ICV99). In addition, the LLNA is internationally validated (Kim95, Kim98, Lov96). This makes the method suitable for deriving dose-effect relations. The latter are in turn suitable for ranking substances by sensitising capacity (Kim92, Bas99, Bas00). Furthermore, the OECD has published a draft guideline relating to the LLNA (OECD00a).

In vitro methods are also emerging. To trace potentially sensitising substances, hopes are being pinned on the determination of various interleukins. Thus, the allergen-specific mediator IL-1β plays an important role during the induction phase of dermal sensitisation. Screening based on this substance can thus provide a great deal of information (Enk92, Enk93). For the performance of this test, cultures of so-called Langerhans' cells must be routinely available (Rom94). Measurement of interleukins released by keratinocytes (for example, IL-18) is also receiving a lot of attention. Yet other methods relate to the migration of Langerhans' cells in combination with the expression of IL-1β and to the determination of cytokines in keratinocytes (Kim94, Ram96). Validation of all these techniques is still at an early stage.

Strategic approach

The Committee advocates the gradual collation of data on dermal sensitisation on the basis of building blocks discussed in the foregoing sections. The following three-stage process has been proposed in the literature (Bas95), although the Committee repeats its general observation that there may be grounds to adopt different decision-making rules.

Phase 1 It is examined via the DEREK system whether the molecule under investigation (or its metabolite) has '*structural alerts*'. If so, phase 2 follows.

Phase 2 Determination of skin penetration capacity via a QSAR model. If this is concerned, it can be assumed that the substance can cause contact dermatitis in humans. If the findings of phases 1 and 2 are negative - in other words, there is no evidence that the substance has skin-sensitising properties - an in vivo test can be performed for confirmation purposes (phase 3).

Phase 3 An LLNA is performed with mice. A positive result indicates a sensitising capacity. In the event of a negative result, the reverse applies and further research can be dispensed with.

Evaluation

The Committee points out that a multitude of molecular and cellular processes are involved in the development of dermal sensitisation. Their relative contributions and interdependence are only partly understood. It is very likely that a combination of in vitro methods will therefore need to be adopted in the long term. It is at any rate too early for this at the present time: these methods are still at the developmental stage and have not yet been validated. Core elements of the assessment of dermal sensitisation are, with the current state of knowledge, certain QSAR models and more recent in vivo techniques such as the LLNA. Anticipating Chapter 4, which examines more general considerations of test strategies, the Committee would also point out that it will depend on the use of a substance which toxicity data is required. Thus, the risk of dermal sensitisation will remain limited to certain employees if an intermediary substance in an industrial production process is involved. Screening on the basis of QSARs may then suffice under certain circumstances.

3.3 Neurotoxicity

In terms of structure and function, the nervous system is one of the most complex organ systems. It coordinates not only the biochemical apparatus behind skills such as learning and memory, but also influences and manages virtually all physiological processes. This complexity contributes to the vulnerability of the whole. The nervous system consists of a wide variety of neurons (nerve cells) and glial cells (supporting cells). Neurotoxicity is any form of harmful effect on the structure and operation of the nervous system as a result of exposure to chemical influences. What changes are to be interpreted as harmful in this context is, however, by no means always clear. Thus, disagreement exists as to the question of whether neurochemical changes without structural damage should be regarded as adverse (Cos98). In practice, toxicologists are focusing on a wide range of phenomena: changes of a morphological, neurochemical, neurological or neuropsychological nature; the extent to which such changes occur; and whether temporary or permanent effects are concerned.

Mechanisms of action

In view of the variety of possible target cells and their complex mutual relationships, it is understandable that there are countless ways in which disruption of and damage to the nervous system can arise (Til92). These include strengthening, weakening or blocking of neurotransmission (nerve impulse conduction) via the disruption of certain stages in the transmission process, primary damage to the cell body or to the branches of nerve cells via an effect on vital functions, and damage to the myelin sheath; the latter plays an important role in the transmission of the nerve impulse. Indirect damage is also possible, for example through damage to what is dubbed the blood-brain barrier, a cellular system that protects the brain against the penetration of unwanted substances from the blood.

The link between structural and functional forms of damage may display considerable variation (Dor00, NRC92). Neuroanatomical changes are generally regarded as adverse (Set92). However, many substances are capable of radically influencing the operation of the nervous system without any perceptible structural damage being involved (Dor00). Purely relying on neuropathological (structural) data for the identification of neurotoxic agents thus does not provide a sufficient guarantee. Although the nervous system can functionally adapt once damaged, the limited recovery capacity of nerve cells quickly limits this possible adaptation. A further complication is that not all parts of the nervous system are equally vulnerable to exposure to neurotoxic substances (Dor00). This variation in vulnerability may be bound up with such varied things as the regional distribution of neurotransmitters, the variation in blood flow, the level of absorption of a particular neurotoxic agent and inherent cellular vulnerability.

Tested methods

Various – proposed or established – procedures exist for assessing the neurotoxicity of substances. These test procedures are to a large extent based on in vivo methods and often have a tiered structure (so-called '*tiered testing*') (EPA98, NRC92, Sob96). The Committee further examines this particular aspect in the section on 'strategic approach'. Here, it lists what kinds of tests are involved. These can be roughly subdivided into four types (Dor00). Neuropathological methods are used to identify structural damage to the nervous system. They represent the 'classic' way of tracing neurotoxicity. Various manuals describe which parts of the nervous system should then be examined and how this should take place (Spe80, IPCS01). An understanding of the molecular and biochemical effects of exposure to substances can be gained via neurochemical means. A wide range of possible end points exist, from influences on

neurotransmitters and their receptors to effects on enzymes that regulate neural activity (NRC92, IPCS01). In the case of a limited number of (groups of) substances, it is known which molecules are the precise target. Thus, the neurotoxic capacity of certain organophosphates can be reduced to inhibition of the activity of the enzyme acetylcholinesterase (NRC92). Electrophysiological techniques enable researchers to assess the functional activity of the nervous system. It has long been known, for instance, that exposure to substances such as lead, hexane and carbon disulfide may adversely affect nerve conduction speed (NRC92). And there is also a battery of behavioural tests allowing cognitive, motor and sensory function to be charted. As with neuropathological methods, the detection of manifest health damage is entailed. One important analytical tool is what is known as the '*Functional Observation Battery (FOB)*'. Various FOBs of this kind have now been developed, all geared to assessing sensory motor function (EPA98, NRC92).

To sum up in a nutshell: the wide range of possible neurotoxic effects can thus be determined using a huge arsenal of methods and techniques. The other side of this coin is, however, that this may take a great deal of time and money. As with other end points, faster, simpler and cheaper analytical techniques are thus being sought for neurotoxicity as well.

Mechanistically oriented methods

Methods for predicting – potential – neurotoxicity on the basis of the physicochemical structure of substances are still at an early stage of development, at least compared with end points such as dermal sensitisation and carcinogenicity (Rid96). Thus, DEREK, the previously mentioned computer program for QSARs, contains 106 'prediction rules' for dermal sensitisation and only 5 for neurotoxicity. Again, this situation is due to the complexity of the nervous system. Neurotoxicity manifests itself in so many ways that it is difficult to rule out the possibility *a priori* of a substance not having neurotoxic properties. And even if the type of neurotoxicity is known, understanding of the precise mechanism of action is generally lacking (NRC92).

Here, too, in vitro procedures can in principle meet desired characteristics such as speed and suitability. Up to now, in vitro tests have mainly been used to clarify the mechanisms of action of neurotoxic agents (Cos98). Such tests have, for example, proved useful in the identification and analysis of so-called excitotoxic substances (NRC92). Since, as mentioned, the nervous system is comprised of a multitude of cell types, it is clear from the outset that a single test cannot suffice to identify all potential effects (Ver92). Depending on what is already known about the neurotoxicity of a substance and the specific questions in which one has a precise interest, various cellular systems are of use (Cos98). Let us take two examples. Neuroblastoma and

glial cells are suitable for investigating the interaction of substances with receptor and signal transduction systems. Via so-called Schwann cells, an understanding can be gained of the possible effects on the myelin sheath. Toxicologists have examined, among other things, the value of certain in vitro tests for screening for (potential) neurotoxic properties of substances. The challenge – and difficulty – lies in differentiating specific forms of neurotoxicity from basic cytotoxicity. The appropriate format for a test battery is still a matter of fierce debate (Cos98, NRC92). Following on from this, attention must also be paid to validation of the proposed procedures.

Strategic approach

Partly owing to the variety of neurotoxic effects, the idea of '*tiered testing*' crops up frequently in the literature (NRC92, OTA90, Sob96, Til92). The proposed procedure, which is followed by various bodies, generally has three stages.

Phase 1 The identification of neurotoxicity. This calls for a sufficiently broad test battery, which may comprise both in vivo and in vitro tests. Judging by current scientific understanding, one cannot, according to the Committee, confine oneself to the latter systems. Further research must show how specific and sensitive screening on the basis of purely in vitro techniques can be in the long term. In accordance with the American EPA's guidelines laid down several years ago, the Committee considers screening with suitable in vivo tests, in particular an FOB, to be indispensable for the time being (EPA98). In vitro techniques may then, if desired, provide additional information on mechanisms of action. In the event of a positive result (i.e. neurotoxicity exists), various options present themselves. For instance, an intended commercial application of the substance in question may be dispensed with. If the substance is, for instance, already in circulation or if its use brings clear benefits, it can be decided to conduct further analysis.

Phase 2 Characterisation of the nature of the neurotoxicity. During this phase, it must be investigated which part of the nervous system proves to be the most important target and how the exposure-effect relationship looks. It is then appropriate, in line with data from the first phase, to select techniques from the huge arsenal of tested methods.

In arriving at decisions on this, it will, in the Committee's view, also be a factor whether the substance under investigation has other toxic properties and what the situation is as regards (potential) exposure to it. Furthermore, various investigators have in recent years advocated paying closer attention than in the past to the neurotoxic damage that may be sustained by children during early development (Cla00, Mil01, Til00).

Phase 3 Clarification of the mechanism of action. According to the various proposals, this test phase arises if, for example, substances with severe effects and broad distribution are involved, such as lead or certain pesticides.

Evaluation

To sum up, the Committee considers that, at least for a complex end point such as neurotoxicity, the selection of tests for phase 1 is of crucial importance. Truly neurotoxic substances will then immediately – and not only later – be recognised as such. And any desired follow-up research will be capable of being shaped in as targeted a fashion as possible. With the current state of understanding, in vivo tests should play a key role during this first phase. In vitro procedures seem, generally speaking, to be relegated to a secondary role for the time being. However, bright points do nonetheless exist. Thus, a prevalidation study carried out on the predictive value of the integration of in vitro data and biokinetic models yielded various interesting findings (Jon99). In the case of eight known neurotoxic substances, it was examined how close the accordingly estimated LOEL (*Lowest Observed Effect Level*) is to the LOEL determined via the in vivo route. The differences proved not to amount to more than a factor of two to ten. The Committee considers such exercises to be useful, but points out that the investigators had the necessary preliminary knowledge. This therefore does not mean in the least that an approach of this kind works well for all substances or all neurotoxic end points. Further research will have to provide a definite answer.

3.4 Carcinogenicity

In the previous chapter, the Committee repeatedly pointed out that carcinogenicity, particularly its genotoxic form, is an end point that now lends itself extremely well to the application of mechanistically oriented analytical techniques. It substantiates this assertion in more detail below.

Mechanisms of action

Carcinogenesis is a complex process in which the accumulation of mutations in specific genes (so-called proto-oncogenes and tumour suppressor organs) plays a key role. Another advisory report from the Health Council of the Netherlands contains detailed examinations of this process (GR96a). Through such mutations, healthy cells change into latent cancer cells. How many mutations are necessary, and which ones, differs for each type of cancer. The causes are various: exposure to specific chemical

substances and to ionising radiation is one culprit, although naturally occurring defects in the replication of DNA during cell divisions are also responsible for the mutations. Most of this induced DNA damage is for that matter eliminated by DNA repair processes.

Carcinogenic substances are usually classified into two categories on the basis of their mechanism of action, namely into ‘genotoxic’ and ‘non-genotoxic’ substances. Genotoxic carcinogens have the effect of damaging DNA, either directly, via a reaction with the DNA – whether or not after metabolic activation – or indirectly, for example by disruption of DNA repair or synthesis (GR96a). The direct variant is the commoner of the two. It is assumed in this context that each level of exposure brings about the damage referred to. In other words, there is no threshold effect. In the case of indirectly acting substances (for example, arsenic and cadmium compounds), a threshold does indeed exist below which no harmful effect occurs.

A threshold of this kind also exists for non-genotoxic carcinogens. With these substances, damage arises via mechanisms other than binding to DNA or interference with DNA repair processes. Here as well two categories can be distinguished: substances that damage the cell and substances that promote cell growth and division. In both cases, the normal processes of growth and differentiation are disrupted, promoting the formation of tumours (But92, Sch98). An organ-specific action is often involved (Far84). So-called tumour-promoting substances also belong to this category of carcinogens (GR96a).

Tested methods

Besides epidemiological research, established methods for detecting possible carcinogenic effects of substances include the following: long-term animal experiments, in vivo genotoxicity tests and in vitro genotoxicity tests.

Regulatory authorities currently accept only long-term animal experiments as a method for determining the risk (expressed in quantitative terms) of exposure to carcinogenic substances (i.e. if there is no or inadequate epidemiological data, which is mostly the case). Mice or rats are normally used for this. However, this approach is increasingly coming under fire (GR96, Sch98). Firstly, there are misgivings about the extent to which these experimental animals represent a good model for humans. In addition, serious doubts have arisen as to the relevance of the findings of such experiments at the usual high dosages.

Only a limited number of in vivo methods enabling genotoxicity to be established have been validated. Most relate to chromosomal changes or to so-called micronucleus induction (CEC96, GR95, Gre00). The Health Council of the Netherlands has recommended two tests: the micronucleus test and the UDF test (for demonstrating

DNA repair) (GR95). According to the Council, a positive outcome to these tests indicates carcinogenicity, although the carcinogenic potency of the tested substance cannot yet be quantified.

The advisory report of the Health Council of the Netherlands just alluded to also contains recommendations on the use of in vitro methods (GR95). The minimum package consists of the following three tests: research on gene mutations in bacteria and in mammalian cells and determination of structural chromosomal aberrations in mammalian cells, in the presence and absence of a metabolic activation system. Other bodies have made similar proposals (CEC96, Gre00). Opinion is divided on the evidential value of the findings. According to the advisory report of the Health Council of the Netherlands, a 'negative score' does not provide an adequate guarantee that the substance has no genotoxic properties. Others consider that it does (Mul99). Generally speaking, the view is that 'positive scores' must be confirmed by in vivo research (GR95, Gre00).

Short-term and validated tests via which non-genotoxic carcinogenicity can be demonstrated are lacking.

Mechanistically oriented methods

'*Structural alerts*' exist which, after any metabolic activation, predict whether a substance reacts with DNA (GR95). The associated (Q)SAR models can sometimes be refined if it is known how binding to DNA comes about (Loh99). Such models have up to now chiefly been important as a first stage in a test strategy, for example when setting priorities for testing a group of substances. In the case of non-genotoxic carcinogens, there is still a shortage of SAR models. This is due to the multitude of possible mechanisms of action.

Over the last twenty years, various methods have been developed for demonstrating and quantifying DNA adducts, particularly in order to clarify the mechanisms of action of genotoxic substances (IARC88, ECE98, IARC93, IARC94). One much-used technique is the so-called 32P post-labelling method. Using this highly sensitive and internationally validated technology, one adduct per cell can even be detected for certain substances (IARC93). Immunochemical and spectrometric techniques are also available for such determinations (Sah95).

It is expected that faster methods may in the relatively short term replace certain in vitro tests with mammalian cells, without the sensitivity and specificity of the underlying analyses needing to suffer. Thus, the determination of structural chromosomal aberrations in mammalian cells will, according to some, be able to be replaced by the micronucleus test (Mil97). Mutagenicity tests in bacterial systems will, however, be retained for the time being (Gre00).

The number of in vivo determinations of genotoxicity has risen sharply in recent years. This is firstly because cytogenetic techniques have been developed whereby hereditary chromosomal characteristics can be determined, such as aneuploidy and stable translocations (Bau99, Eas94). Secondly, methods are available for measuring mutations in experimental animals (whether or not transgenic). Particularly the determination of so-called *Hprt* mutations in lymphocytes has experienced rapid development (Gre00, Str79, Tat98). According to research, an increased frequency of such mutations arises after relatively low exposure to genotoxic substances and ionising radiation (Tat99, Wal99). The occurrence of these mutations is an indicator of the induction of mutations in oncogenes in other tissues (Jan95, Man96). The *Hprt* test may thus play an important role in the routine assessment of genotoxicity. In certain cases, the test also provides scope for quantitative risk analyses (Sit00a). Yet another field of application is monitoring, for example monitoring of workers who come into contact with genotoxic substances. Less highly developed, but offering equally good prospects, is the so-called thymidine kinase (tk) test, via which mutations in autosomal genes are determined (Dob99).

As stated, more uncertainty exists on non-genotoxicity. A wide variety of changes in gene expression is involved here as a result of changes in intracellular signal transduction and interactions with receptors (Pit95). Examples are the so-called Ah receptor for binding with PCBs, the peroxisome proliferator receptor and the oestrogen receptor (ECE92, Sch95). Tests for demonstrating the interactions in question must quickly be substance-specific. One general method is the measurement of cell replication, a possible effect associated with a cytotoxic action (But92, ECE91).

Lastly, transgenic animal models are emerging. These may serve various purposes. Thus, spontaneous and induced mutations can be directly determined in tissues via these models (Gos98, Hed00). This may be of great value both in clarifying mechanisms of action and in quantifying risks. Examples in this connection are mice and rats in which certain bacterial genes have been incorporated (Bur93, Noh96). The OECD is engaged in drawing up guidelines for the application referred to here. Furthermore, alternatives to the mice used in conventional long-term testing have been developed (Ten98). These concern animal models with proto-oncogenes, tumour suppressor organs or DNA repair genes that have been modified in such a way as to have greater sensitivity to carcinogenic substances (Lee94, Ten95, Ten98, Vri97, Yam97). During a validation study, the so-called *p53*, *rasH2* and *XPA* models have up to now turned out to be the most usable. However, it is still far from clear what the results of research on transgenic mice of this kind say about risks for humans.

Before the Committee describes how a strategic assessment of carcinogenicity can take shape, it would emphasise that understanding of mechanisms of action is sometimes sufficiently detailed and complete for direct application in risk analyses.

Let us consider a few examples. Exposure to methylene chloride leads, in mice, to tumours of the liver. In vitro and in vivo research shows that such tumours are the result of reactive metabolites that are formed only in mice and which result in so-called DNA protein '*cross-links*'. The animal experiment data are thus irrelevant here for humans (Cas96).

Besides such qualitative differences, differences of a quantitative nature exist. Exposure to formaldehyde is also accompanied by the formation of DNA protein '*cross-links*', in this case in experimental animals and humans alike. The risk of nasal tumours consequently rises. However, adduct formation at low levels of exposure is disproportionately much more modest than at the high dosages characteristic of long-term animal experiments. Linear extrapolation on the basis of findings at high doses then leads to an overestimation of the risk at low exposure levels. According to calculations, roughly one order of magnitude is involved (Con95, Moo99).

Research shows that mice are more sensitive than rats when exposed to 1,3-butadiene. This difference in sensitivity proves to be connected with a difference in the quantity of DNA adduct formed following exposure. Comparison with data on adduct formation in occupationally exposed people shows that the risk for humans is lower than for rats and much lower than for mice (Sit00b).

Strategic approach

Over the last few decades, innumerable proposals have been made for a stepped assessment of the possible mutagenicity and genotoxic carcinogenicity of substances (Bri74, But92, ECE87, Gre00, Sch98). Both in vitro and in vivo methods formed part of the proposed strategies from an early stage. As stated, the available stock of analytical techniques has, however, grown substantially over time. The Committee outlines here how a test strategy might look, with the corollary that other stages and decision-making rules may also be adopted, as is apparent from various current procedures.

Phase 1 Application of SARs. If 'structural alerts' exist for reactivity with DNA (whether or not after metabolic activation), phase 2 is initiated. If not, it must be examined on a case-by-case basis whether phase 1 suffices. The use of the substance under investigation and the expected exposure profile are important criteria during this appraisal.

Phase 2 In vivo measurement of DNA adducts via the 32P post-labelling method. In the event of a positive outcome, it may be assumed that the substance is potentially genotoxic. This is investigated in greater detail in phase 3. In the event of a negative

result, it must be considered, again on a case-by-case basis, whether further research is unnecessary.

Phase 3 Performance of in vitro mutagenicity tests. The package to be recommended consists of three tests: one test for demonstrating gene mutations in bacteria, one for demonstrating gene mutations in mammalian cells and the micronucleus test in mammalian cells, each performed in the presence and absence of a metabolic activation system. If all three tests are positive, one moves on to phase 4. If all three are negative, it may be duly assumed that the substance does not possess genotoxic properties. Further testing is then unnecessary. In cases in between, a weighted decision is appropriate.

Phase 4 Performance of mutagenicity tests on experimental animals. The *Hprt* test and the micronucleus test in bone marrow cells or in lymphocytes may be used. If a double positive is produced, it is certain that the substance has a genotoxic action in experimental animals. Phase 5 is then necessary to investigate what the findings say about expected effects in humans. If both tests are negative, further research may be dispensed with. In the event of one positive result, a weighted decision must be taken.

Phase 5 Research on the mechanism of action. This may entail in vitro or in vivo research on the biokinetics of the substance and on the formation of DNA adducts in various organs. Thus, qualitative or quantitative interspecies variations can be traced. If the findings for experimental animals are relevant for humans, phase 6 follows. Otherwise, the research can be stopped.

Phase 6 Performance of a long-term animal experiment. On the strength of the data collected in phase 5, the experimental animals closest to humans in terms of biokinetics and toxicodynamics is chosen. If tumours occur, phase 7 is initiated. If not, the substance is regarded as non-carcinogenic.

Phase 7 Quantification of the risk. Information on the substance’s mechanism of action gained during earlier phases is again to be utilised here.

Evaluation

Relatively speaking, a great deal is known about the mechanisms of action of genotoxic carcinogenicity. Various analytical methods adopted in toxicology may thus be applied here if they have not already acquired a clear niche for themselves. However, certain methods, such as the use of transgenic animal models, must still be

further validated first. Understanding of mechanisms of non-genotoxic carcinogenicity is more limited.

Chapter 4

Strategic guidance

There are various reasons for the choice of the word ‘guidance’ in the title of this chapter. Guidance is designed to provide a basis for decisions, but is also meant to allow a certain measure of scope for variation in the course of action to be followed. The need for this exists particularly when all kinds of uncertainties apply to the interpretation of toxicological data, something with which toxicologists often have to deal. Furthermore, the Committee has already remarked in the introductory chapter that demand for toxicity data relating to various types of substances (such as industrial chemicals, pharmaceutical products, pesticides and food additives) may differ. Risk-benefit appraisals will as a rule play a part in this. A differentiated approach is also advocated in the recently published ‘*Strategy memorandum on dealing with substances*’ (VROM01) and in the memorandum on a ‘*Strategy for a future chemicals policy*’ drawn up by the EU (CEC01). All this generally calls for the situation-specific formulation and application of more general approaches. Certain tests may then, for example, receive special attention. Although the Committee, as it made clear in Chapter 1, is advocating better implementation of new toxicological understanding, and more specifically in terms of decision-making, it does not have in mind a compulsory protocol. Rather, it has in mind a guideline for experts who have to make a judgment about the toxicity of a substance, or the risk concerned, in specific contexts.

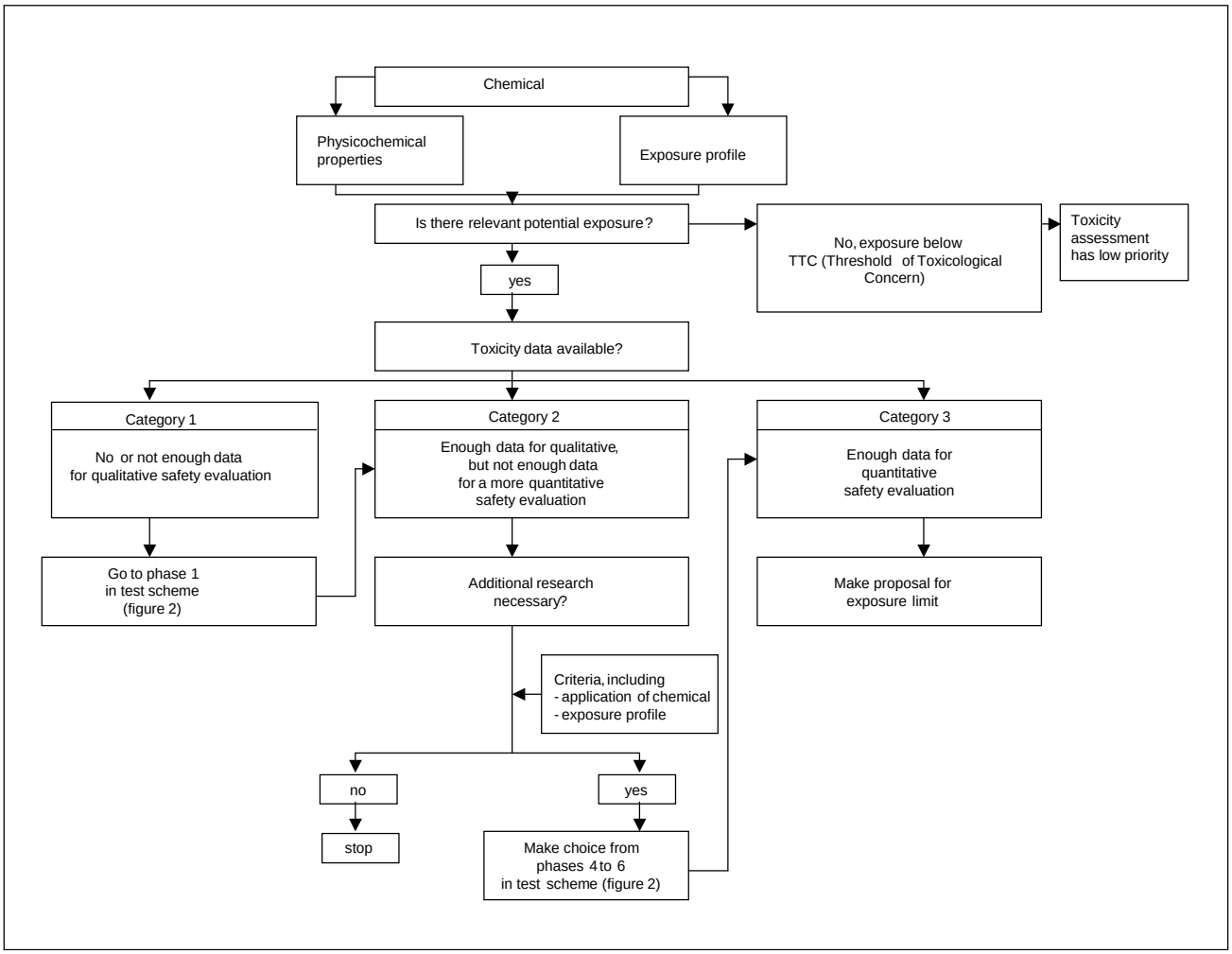

Figure 1 Substances and theis toxicity profile.

Stages and rules

The variability just alluded to has already emerged from the four examples given in the previous chapter. Test schedules for other end points, such as dermal irritation, phototoxicity, immunotoxicity, hormonal disruption and developmental and reproductive toxicity, further supplement this picture (ECE90, EPA00, IPCS94, NRC00, Spi94). In abstract terms, and considered from the structural point of view, there are various aspects that are open to choice. Thus, the number of '*tiers*' can, to begin with, be chosen. Furthermore, it is possible to make serial or parallel links: are certain information sources considered step-by-step or, conversely, at the same time? Choices relating to this question are reflected in the characteristics of the tiers: do they

contain few or, conversely, many elements? And then there is the nature of the decision-making rules to be adopted: what criteria count and is, for example, a weight-of-evidence approach adopted or not?

The Committee has in Chapter 1 already touched on what considerations it is guided by. Information on the exposure profile is one of these criteria. Complexity – and thus costs – of methods is another. In accordance with the developments and views described in previous chapters, simpler analytical techniques or analytical techniques geared to lower levels of biological organisation (such as '*high throughput*' systems and certain in vitro methods) will, in the Committee's view, have to come before more specialised, but also more expensive methods (mostly in vivo studies). This can also be expressed as follows: first, a more qualitatively oriented process is needed in which attention is chiefly focussed on (possible) toxicity mechanisms. Should the findings together with such things as the exposure profile so dictate, a more quantitative analysis can then be performed, in line with information obtained earlier. If desired, this may result in a detailed risk assessment. Strategic flexibility will in any event be absolutely crucial.

Decision-making proposal

The Committee will illustrate its ideas on strategic analyses with the aid of two flow diagrams (shown in Figures 1 and 2). First, a comment on terminology. Up to now, the terms 'toxicity assessment' and 'risk assessment' have consistently been used. In Chapter 1, the Committee has described in overall terms what is generally understood by these terms. Here, it prefers a different term with a similar conceptual content and which is gaining in popularity, namely '*safety evaluation*'. As is apparent from the diagrams, such safety evaluations may differ in comprehensiveness. The Committee then refers to different 'levels' of evaluation. The term 'safety evaluation' expresses in any event the fact that the same objective is always intended, namely protection of public health, irrespective of whether classifications and labelling (safety evaluations of a 'low' level) or the derivation of recommended values and precise risk analyses (involving a 'high' level) are concerned.

Furthermore, the Committee again draws attention to what it alluded to in the previous section: that the proposed phases and binary decision-making rules (yes/no) chiefly serve a pragmatic end and are not absolute in nature. A no does not, for instance, automatically spell an end to the matter. Rather, it should be taken to mean that a more in-depth evaluation – for the time being – has low priority.

As the Committee has argued several times, the collection of toxicity data must, as it sees things, partly be carried out on the basis of exposure information. In fact, this is

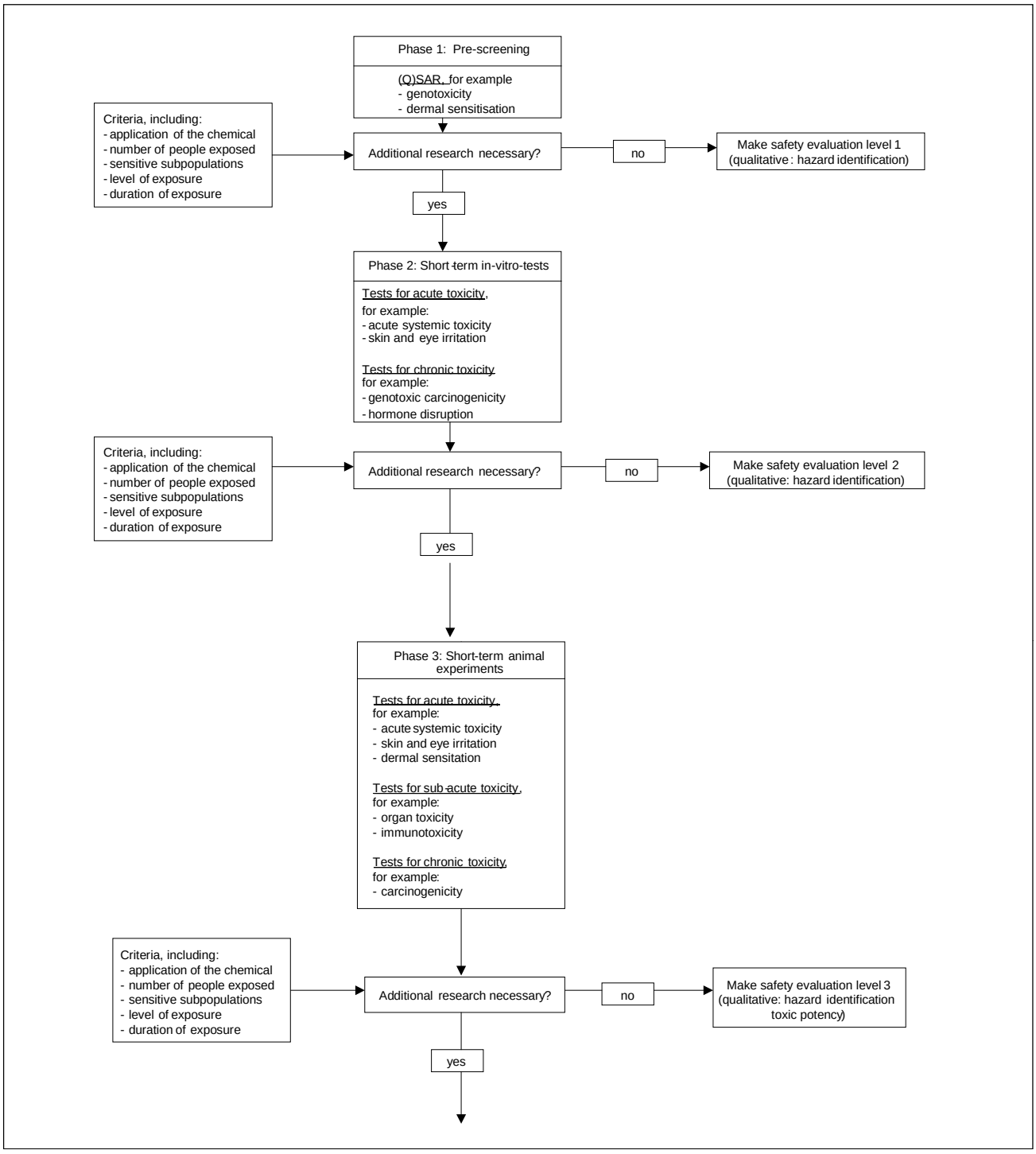

Figure 2 Indicative flow chart for determining the toxicity profile of a chemical.

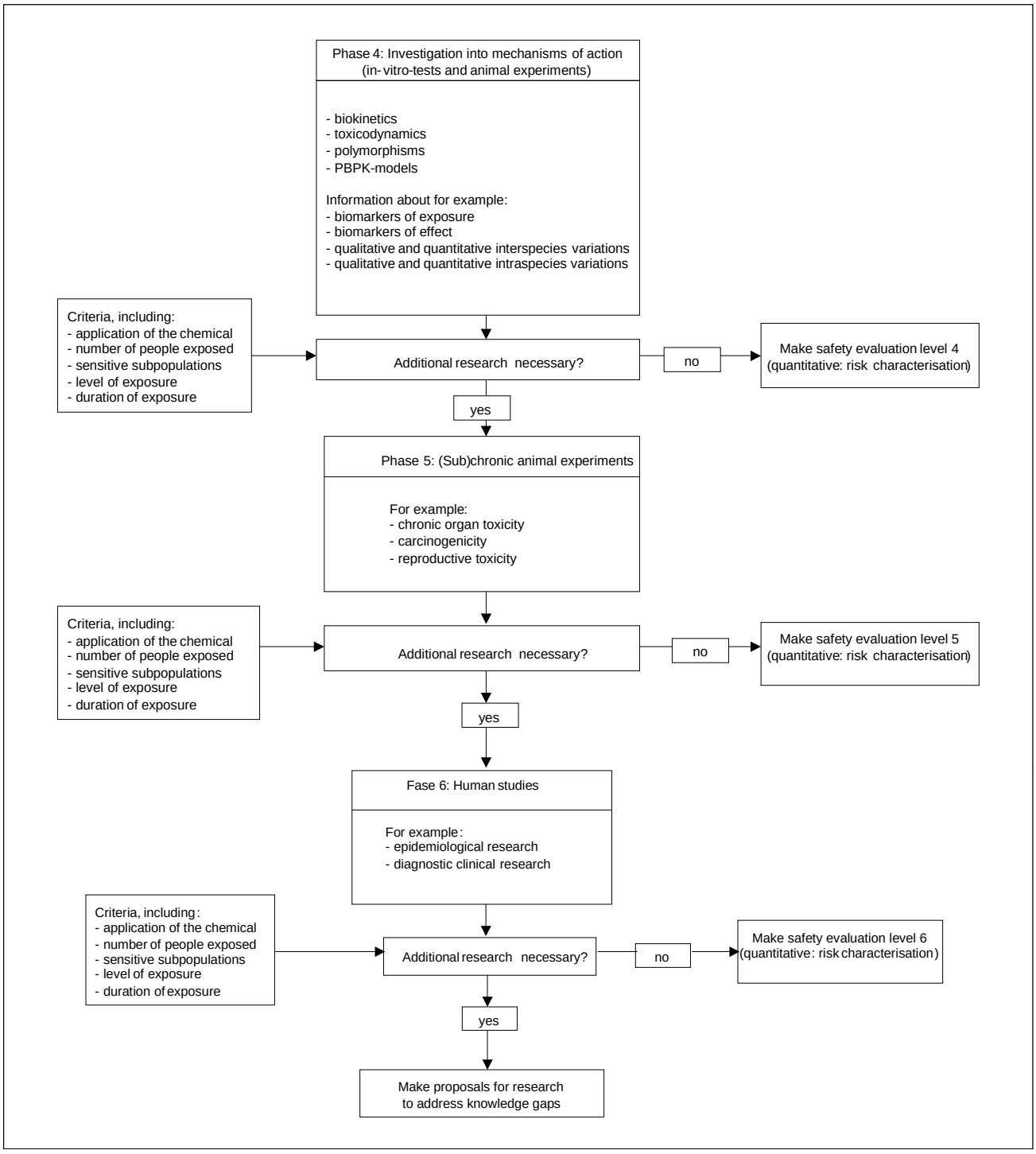

Figure 2 Continued.

a separate field of research and analysis. The Committee does not consider developments in this field in further detail, but confines itself to a few observations of a more general nature. With an exposure analysis, all kinds of aspects will apply, depending on the policy context (in which the benefit of and need for a substance may also come up for discussion). Thus, the (expected) level of individual exposure to the substance is always important. However, the number of people exposed, who is exposed (for example, employees, certain consumers or the entire population) and the duration of exposure may also be factors of variable importance. In both flow diagrams, such exposure characteristics thus occupy a prominent position.

This is clearest from Figure 1: here, the first decision-making stage relates to the question of whether or not relevant (potential) exposure to the substance under consideration is concerned. A more detailed definition of 'relevant' is in part a policy matter. Recently, during the assessment of food additives, a term has cropped up which, according to the Committee, provides a basis for the required process of operationalisation. The term in question is the so-called '*Threshold of Toxicological Concern*' (TTC) (Kro00, Mun98, Mun99). The operationalisation in question encompasses a comparison with data on toxicity for a large number of other substances, more particularly with the so-called NOAELs of these substances (*NOAEL: No Observed Adverse Effect Level*). By charting the distribution of such NOAELs, information is, according to supporters of this approach, obtained on the marginal area between 'safe' and 'unsafe' levels of exposure or, in other terms, on a TTC.

Various possible variants are reviewed in the most recent publication (Kro00). Thus, allied substances can be compared with one another in terms of physicochemical structure. It is also possible to compare on one or more end points. If the expected intake of a substance is below such a TTC, it may be decided to dispense with a further safety evaluation, or to assign it low priority. The TTC method is, in the Committee's view, in any event more meaningful than the rigid links often laid down between production volumes of substances and toxicity data to be supplied. However, in line with the tenor of this advisory report, it will need to be examined for each policy context or each end point how the TTC method can best be formulated or what its limits should be. Particularly for genotoxicity, for which no threshold dose can be determined, this calls for further elaboration.

If toxicologically relevant exposure is involved, a subsequent decision-making stage arises. The Committee distinguishes three possibilities. Category 1 contains substances on which no or insufficient toxicity data is available. New substances belong to this category and generally also chemicals with a low production volume (10-1000 tonnes per annum). If a safety evaluation is desired, information must then be generated. This takes place in accordance with the flow chart in Figure 2. When phases

1, 2 or 3 are passed through, chiefly qualitative information on toxicity is obtained. The substance under investigation consequently falls in category 2. Qualitative safety evaluation, for example labelling or classification in a hazard class, is then possible. Most existing substances with a high production volume (more than 1000 tonnes per annum) belong to this category. If there is a need for higher levels of safety evaluation, i.e. quantitative assessments, phases 4, 5 or 6 are considered. Substances for which such information is available are classified in category 3. Examples include pesticides and certain industrial chemicals with a high production volume. As from phase 3, the possibility arises of deriving recommended limits.

The scheme presented by the Committee in Figure 2 should first and foremost be regarded as an orderly procedure for collecting toxicity data. The available information must in this context determine the focus of further research at every turn. As more phases are passed through, the toxicity profile gains in evidential value. Each of these phases corresponds to a level of safety evaluation, varying from an assessment of the potential, intrinsic toxicity ('*hazard identification*') to comprehensive risk appraisal. Which evaluation level is considered depends on two things: the available and the required data. Regulatory authorities will be able to provide clarity on this particular aspect, in consultation with experts and interested parties and taking account of such things as the application of a substance and the associated exposure profile (the 'criteria').

The flow chart provides scope for new and existing methods. In addition, the description of the phases and components has deliberately been kept so general that future techniques can be easily incorporated. The Committee briefly elucidates the phases, with reference to what has been discussed elsewhere in this advisory report.

Prior to this, the Committee also points out that the exposure analysis set out in Figure 2 under the 'criteria' will in principle become more detailed with increasing levels of toxicological analysis.

Phase 1, in which (Q)SARs are central, is in only a few cases currently sufficient for a safety evaluation. As the Committee pointed out in Chapter 3, dermal sensitisation, mutagenicity or genotoxic carcinogenicity are then above all involved. A 'positive' outcome in this phase may, for example, provide grounds for withdrawing a substance for further development. If '*high throughput*' systems expand enormously, these will also be capable of being accommodated here.

In phase 2, short-term in vitro tests make their appearance. These may shed light on acute or chronic toxicity. In the case of most end points, ongoing research programmes are involved. Validated methods are available for mutagenicity, genotoxic carcinogenicity, dermal irritation and eye irritation.

In phase 3, short-term animal experiments are carried out. Based on the results of these, an initial idea can be gained of exposure-effect relationships. Phase 3 is thus at the interface between qualitatively and quantitatively oriented safety evaluations. Methods for determining genotoxic carcinogenicity are here again the furthest developed. Owing to the lack of validated methods for the phases discussed up to now, long-term animal experiments will for the time being still often have to be utilised (phase 5).

Phase 4, under which methods relating to biokinetics and toxicodynamics have been subsumed, may in this context play a pivotal role, according to the Committee. Biomarkers of exposure and effect, among other things, can in this way be traced. These may be of great use in the setting up of both short-term (phase 3) and longer-lasting (phase 5) studies. Research on humans (phase 6) may also benefit from this. Furthermore, data from phase 4 may reveal what is necessary with respect to interspecies and intraspecies variations and differences between high and low exposure levels. Should pressing questions still remain in connection with phase 6, targeted additional research can be carried out.

Epilogue

The Committee repeats what it stressed at the beginning of this chapter: the flow diagrams provide assistance and do not represent a straitjacket. Depending on circumstances, phases can be skipped or, conversely, an iterative approach may be adopted, as the Committee wanted to illustrate with the comment on the pivotal function of phase 4.

How closely flow diagram 2 can be followed depends on the availability of the respective analytical methods and the extent to which these have been validated. The Committee recommends that this validation be facilitated and promoted at international level.

That experts will have to assess how the gradual construction of a toxicity profile can best proceed does not, for the Committee, mean that there is nothing more to it. As with the current assessment system, it is desirable to record and harmonise all manner of modules and decision-making rules, on an item-by-item basis and according to context. Flexibility and standardisation are not mutually exclusive; on the contrary, in the Committee's view, they represent two parts of the same thing.

References

And87 Andersen ME, Clewell HJ, Gargas, *et al*. Physiologically based pharmacokinetics and the risk assessment process for methylene chloride. Toxicol Appl Pharmacol 1987; 87: 185-205.

And94 Andersen ME, Krishnan K. Physiologically based pharmacokinetics and cancer risk assessment. Environ Health Perspect 1994; 102: 103-8.

Bar94 Barratt MD, Basketter DA, Chamberlain M, *et al*. Development of an expert system rule-base for identifying contact allergens. Toxicol *in vitro* 1994; 8: 837-9.

Bar95 Barratt MD. Quantitative structure activity relationships for skin permeability. Toxicol *in vitro* 1995; 9: 27-37.

Bar98a Barratt MD. Integration of QSAR and in vitro toxicology. Environ Health Perspect 1998; 106: 459-66.

Bar98b Barratt MD. Integrating computer prediction systems with *in vitro* methods towards a better understanding of toxicology. Toxicol Lett 1998; 102-103: 617-21.

Bas95 Basketter DA, Scholes EW, Chamberlain M *et al*. An alternative strategy to the use of guinea pigs for the identification of skin sensitization hazard. Food Chem Toxicol 1995; 33: 1051-6.

Bas96 Basketter DA, Barratt MD, Chamberlain M, *et al*. Identification and classification of chemicals causing skin irritation: a strategy to replace animal tests. ATLA 1996; 24: 233.

Bas99 Basketter DA, Lea LJ, Dickens A, *et al*. A comparison of statistical approaches to the derivation of EC3 values from local lymph node assay dose resonses. J Appl Toxicol 1999; 19: 261-6.

Bas00 Basketter DA, Blaikie L, Dearman RJ, *et al.* Use of the local lymph node assay for the estimation of relative contact allergenic potency. Contact Derm 2000; 42: 344-8.

Bau99 Baumgartner A, van Hummelen P, Lowe XR, *et al*. Numerical and structural abnormalities detected in human sperm with a combination of multicolor FISH assays. Environ Molec Mutagen 1999; 33: 49-58.

Ber91 Bernard A, Lauwerys R. Proteinuria : changes and mechanisms in toxic nephropathies. Crit Rev Toxicol 1991; 21: 373-405.

Bol01 Bolhuis PA. Het belang van farmacogenetica. Ned Tijdschr Geneeskd 2001; 145: 15-8.

Bon95 Bond JA, *et al*. Epidemiological and mechanistic data suggest that 1,3-butadiene will not be carcinogenic to humans at exposures likely to be encountered in the environment or workplace. Carcinogenesis 1995; 16: 165-71.

Boo00 Boogaard PJ, de Kloe KP, Bierau J, *et al*. Metabolic inactivation of five glycidyl ethers in lung and liver of humans, rats and mice in vitro. Xenobiotica 2000; 30: 485-502.

Bor93 Borghoff S. á2u-globulin-mediated male rat nephropathy and kidney cancer: relevance to human risk assessment. CIIT activities 1993; 13: No 4.

Bri74 Bridges BA. The three-tier approach to mutagenicity screening and the concept of radiation-equivalent dose. Mutat Res 1974; 26: 335-40.

Bue65 Buehler EV. Delayed contact hypersensitivity in the guinea pig. Arch Dermatol 1965; 91: 171-7.

Bur93 Burkhart JG, Malling HV. ENU-induced mutagenesis at a single A:T base pair in transgenic mice containing öX174. Mutat Res 1993; 292: 69-81.

But92 Butterworth BE, Eldridge SR. A decision tree approach for carcinogenic risk assessment: application to 1,4-dichlorobenzene. CIIT activities 1992; 12: no's 11-12.

Cas96 d'A Casanova M, Heck H. Of mice, men and methylene chloride. CIIT activities 1996; 16: no 4.

CEC92 Commission of the European Communities. Council Directive of 30 April 1992 amending for the 7th time Council Directive 67/548/EEC on the approximation of laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances (92/32/EEC). Off J Eur Commun L154/1.

CEC93a Commission of the European Communities. Council Regulation (EEC) No 793/93 of 23 March 1993 on the evaluation and control of the risks of existing substances. Off J Eur Communities, L154/1.

CEC93b Commission of the European Communities. Commission Directive 93/67/EEC of 20 July 1993, laying down the principles for the assessment of risks to man and the environment of substances notified in accordance with Council Directive 67/548/EEC. Off J Eur Communities, L227.

CEC96 Commission of the European Communities. Technical guidance documents in support of the Commission Directive 93/67/EEC on risk assessment for new substances and the Commission Regulation (EC) No 1488/94 on risk assessment for existing substances. Brussels: EC, 1996.

CEC01 Commission of the European Communities. White paper. Strategy for future chemicals policy. Preliminary version, subject to editorial adaptations. Brussels: EC, 2001.

CII98 Chemical Industry Institute of Toxicology. The chemical industry's research initiative and the state of the science study. Research Triangle Park: CIIT, 1998.

CII00 Chemical Industry Institute of Toxicology. Long-range research initiative of the American Chemistry Council. Research Triangle Park: CIIT, 2000.

Cla00 Claudio L, Kwa WC, Russell AL. Testing methods for developmental neurotoxicity of environmental chemicals. Toxicol Appl Pharmacol 2000; 164: 1-14.

Cle96 Clemedson C, Mc Farlane-Abdulla E, Anderson E, *et al*. MEIC evaluation of acute systemic toxicity. Part II. In vitro results from 68 toxicity assays used to test the first 30 reference chemicals and a comparative cytotoxicity analysis. ATLA 1996; 24 (Suppl 2): 273-311.

Con95 Conolly RB, Andjelkovich DA, Casanova M, *et al*.. Multidisciplinary, iterative examination of the mechanism of formaldehyde carcinogenicity: the basis for better risk assessment. CIIT activities 1995; 15: no 12.

Cor99 Corton JC, Anderson SP, Stauber AJ, *et al*. Entering the era of toxicogenomics with DNA microarrays. CIIT activities 1999; 19: no 2.

Cos98 Costa LG. Neurotoxicity testing: a discussion of in vitro alternatives. Environ Health Perspect 1998; 106 : 505-10.

Cur98 Curren RD. In vitro alternatives for ocular irritation. Environ Health Perspect 1998: 106: 485-92.

Dob99 Dobrovolsky VN, Casciano DA, Heflich RH. Tk mouse model for detecting in vivo mutation in an endogenous autosomal gene. Mutat Res 1999; 423: 125-36.

Dor00 Dorman DC. An integrative approach to neurotoxicology. CIIT activities 2000; 20 no 3.

Eas94 Eastmond DA, Rupa DS, Hasegawa LS. Detection of hyperdiploidy and chromosome breakage in interphase human lymphocytes following exposure to the benzene metabolite hydroquinone using fluorescence in situ hybridisation with DNA probes. Mutat Res 1994; 322: 9-20.

ECE87 European Centre for Ecotoxicology and Toxicology of Chemicals. Assessment of mutagenicity of industrial and plant protection chemicals. Brussels: ECETOC, 1987; (Monograph No 9).

ECE88 European Centre for Ecotoxicology and Toxicology of Chemicals. Eye irritation testing. Brussels: ECETOC, 1988; (Monograph No 11).

ECE89 European Centre for Ecotoxicology and Toxicology of Chemicals. DNA and protein adducts: evaluation of their use in exposure monitoring and risk assessment. Brussels: ECETOC, 1989; (Monograph No 13).

ECE90a European Centre for Ecotoxicology and Toxicology of Chemicals. Skin irritation. Brussels: ECETOC, 1990; (Monograph No 15).

ECE90b European Centre for Ecotoxicology and Toxicology of Chemicals. Skin sensitisation testing. Brussels: ECETOC, 1990; (Monograph No 14).

ECE91 European Centre for Ecotoxicology and Toxicology of Chemicals. Early indicators of non-genotoxic carcinogenesis. Brussels: ECETOC, 1991; (Monograph No 16).

ECE92 European Centre for Ecotoxicology and Toxicology of Chemicals. EC 7th Amendment: Role of mammalian toxicokinetic and metabolic studies in the toxicological assessment of industrial chemicals. Brussels: ECETOC, 1992; (Technical Report, No 46).

ECE92b European Centre for Ecotoxicology and Toxicology of Chemicals. Hepatic peroxisome proliferation. Brussels: ECETOC, 1992; (Monograph No 17).

ECE93 European Centre for Ecotoxicology and Toxicology of Chemicals. Percutaneous absorption. Brussels: ECETOC, 1993; (Monograph No 20).

ECE96 European Centre for Ecotoxicology and Toxicology of Chemicals. Risk assessment for carcinogens. Brussels: ECETOC, 1996; (Monograph No 24).

ECE99 European Centre for Ecotoxicology and Toxicology of Chemicals. Long-range research Initiative (LRI). Brussels: ECETOC, 1999; (Human and Environmental Health Research Proposals).

ECE00 European Centre for Ecotoxicology and Toxicology of Chemicals. Skin sensitisation testing for the purpose of hazard identification and risk assessment. Brussels: ECETOC, 2000; (Monograph No 29).

Enk92 Enk AH, Katz SI. Early molecular events in the induction phase of contact sensitivity. PNAS 1992; 89: 1398-1402.

Enk93 Enk AH, Angeloni VL, Udey MC, *et al.* An essential role for Langerhans cell-derived IL-1 ß in the initiation of primary immune responses in skin. J Immunol 1993; 150: 3698-3704.

EPA98 U.S. Environmental Protection Agency. Guidelines for neurotoxicity risk assessment. Washington: EPA, 1998.

EPA00 U.S. Environmental Protection Agency. Endocrine disruptor screening programme. Report to congress. Washington: EPA, 2000.

Far84 Mac Farland HN. Xenobiotic induced kidney lesions: hydrocarbons, the 90 days and 2-year gasoline study. In: MA Mehlman, GP Hemstreet, JJ Thorpe, *et al*., red. Renal Effects of Petroleum Hydrocarbons. Princeton, 1984 : 51-6.

Far99 Farr S. Concise review: gene expression applied to toxicology. Toxicol Sci 1999; 50 : 1-9.

Fau99 Faustman EM, Lewandowski T, Ponce R, *et al.* Biologically based dose-response models for developmental toxicants: lessons from methylmercury. Inhal Toxicol 1999; 11: 101-14.

Fed95 Fedtke N. Extrapolation of toxicity data and assessment of risk. In: Thomas H, Hess R, Waechter F, red. Toxicology of industrial compounds. Toxicology of industrial compounds. London: Taylor & Francis, 1995: 173-84.

For95 Forsby A, Pilli F, Bianchi V *et al*. Determination of critical cellular neurotoxic concentrations in human neuroblastoma (SH-SY5Y) cell cultures. ATLA 1995; 23: 800-11.

Fre93 Frederick CB. Limiting the uncertainty in risk assessment by the development of physiologically based pharmacokinetic and pharmacodynamic models. Toxicol Lett 1993; 68: 159-75.

Gad86 Gad SC, Dunn BJ, Dobbs DW, *et al*. Development and validation of an alternative dermal sensitisation test: the mouse ear swelling test (MEST). Toxicol Appl Pharmacol 1986; 84: 93-114.

Gol98 Goldberg AM, Maibach HI. Dermal toxicity: alternative methods for risk assessment. Environ Health Perspect 1998; 106 : 493-6.

Gon98 Gonzales FJ. The study of xenobiotic-metabolizing enzymes and their role in toxicity in vivo using targeted gene disruption. Toxicol Lett 1998; 102-103: 161-6.

Gos89 Gossen JA, de Leeuw WJ, Vijg J. LacZ transgenic mouse models: Their application in genetic toxicology. Mutat Res 1989; 307: 451-9.

GR95 Gezondheidsraad. Betekenis van mutageniteitstests. Den Haag: Gezondheidsraad, 1995; publicatie nr 1995/20.

GR96 Gezondheidsraad. Toxicologische advieswaarden voor blootstelling aan stoffen. Den Haag: Gezondheidsraad, 1996; publicatie nr 1996/12.

GR96a Gezondheidsraad. Beoordeling carcinogeniteit van stoffen. Den Haag: Gezondheidsraad, 1996; publicatie nr 1996/26.

GR98 Gezondheidsraad. HELLE: Health effects of low level exposures. Den Haag: Gezondheidsraad 1998, publicatie nr 1998/18.

Gra94 Grandjean P, Brown SS, Reavy P *et al*. Biomarkers of chemical exposure: state of the art. Clin Chem 1994; 40: 1360-2.

Gre00 MacGregor JT, Casciano D, Müller L. Strategies and testing methods for identifying mutagenic risks. Mutat Res 2000; 445: Chapter 1.

Gui98 Guillouzo A. Liver cell models in in vitro toxicology. Environ Health Perspect 1998; 106: 511-32.

Gul94 Gülden M, Seibert H, Voss JU. Inclusion of physicochemical data in quantitative comparisons of in vitro and in vivo toxic potencies. ATLA 1994; 22: 185-92.

Hed00 Heddle JA, Dean S, Nohmi T, *et al.* In vivo transgenic mutation assays. Environ Mol Mutagen 2000; 35: 253-9.

Hor92 Horton VL, Higuchi MA, Rickert DE. Physiologically based pharmacokinetic model for methanol in rats, monkeys and humans. Toxicol Appl Pharmacol 1992; 117: 26-36.

IARC88 International Agency for Research on Cancer. Methods for detecting DNA damaging agents in cancer epidemiology and prevention. Lyon: IARC, 1988; (IARC Scientific Publications No 89).

IARC93 International Agency for Research on Cancer. Postlabelling methods for detection of DNA adducts. Lyon: IARC, 1993; (IARC Scientific Publications No 124).

IARC94 International Agency for research on cancer. DNA adducts: identification and biological significance. Lyon: IARC, 1994; (IARC Scientific Publications No 125).

ICV99 Interagency Coordinating Committee for the Validation of Alternative Methods. The murine local lymph node assay: a test method for assessing the allergic contact dermatitis potential of chemicals/compounds. Washington: NIH, 1999; (Report NIH No 99-4494).

IPCS94 International Programme on Chemical Safety. Principles and methods for assessing direct immunotoxicity associated with exposure to chemicals. Genève: WHO, 1994.

IPCS01 International Programme on Chemical Safety. Neurotoxicity risk assessment for human health: principles and approaches. Genève: WHO, 2001.

Jac99 Jackson TE, Schlosser PM, Medinsky MA, *et al*. Assessing the role of CYP2E1 in the metabolism and genotoxicity of 1,3-butadiene. Toxicol Sci 1999; 48 (1-S); Abstract 518.

Jan95 Jansen JG, de Groot AJL, van Teijlingen CMM, *et al*. Induction of hprt gene mutations in splenic T-lymphocytes from rats exposed in vivo to DNA methylating agents is correlated with formation of O6-methylguanine in bone marrow and not in the spleen. Carcinogenesis 1996; 17: 2183-91.

Joh93 Johanson G, Filser JG. A physiologically based pharmacokinetic model for butadiene and its metabolite butadiene monoxide in rat and mouse and its significance for risk extrapolation. Arch Toxicol 1993; 67: 151-63.

Jon99 de Jongh J, Forsby A, Houston JB, *et al*. An integrated approach to the prediction of systemic toxicity using computer-based biokinetic models and biological in vitro test methods: overview of a prevalidation study based on the ECITTS project. Toxicol in vitro 1999; 13: 549-54.

Kar98 Karol MH. Target organs and systems: methodologies to assess immune system function. Environ Health Perspect 1998; 106: 533-40.

Kim94 Kimber I. Cytokines and regulation of allergic sensitisation to chemicals. Toxicology 1994; 93: 1-11.

Kim97 Kimber I, Basketter DA. Contact sensitisation : a new approach to risk assessment. Human Ecol Risk Assess 1997; 3: 385-95.

Kim98 Kimber I, Hilton J, Dearman RJ, *et al.* Assessment of the skin sensitisation potential of topical medicaments using the local lymph node assay: an inter-laboratory evaluation. J Toxicol Environ Health 1998; 53: 563-79.

Kim99 Kimber I, Gerberick GF, Basketter DA. Thresholds in contact sensitisation: theoretical and practical considerations. Fd Chem Toxic 1999; 37: 553-60.

Kri92 Krishnan K, Gargas ML, Fennell TR, *et al*. A physiologically based description of ethylene oxide dosimetry in the rat. Toxicol Ind Health 1992: 8: 121-40.

Kro00 Kroes R, Galli C, Munro I, *et al*. Threshold of toxicological concern for chemical substances present in the diet: a practical tool for assessing the need for toxicity testing. Fd Chem Toxic 2000; 38: 255-312.

Lau00 Lau C, Andersen ME, Douglas J, *et al*. Evaluation of biologically based dose-response modelling for developmental toxicity: a workshop report. Regul Toxicol Pharmacol 2000; 31: 190-9.

Lee94 Lee JM, Abrahamson JL, Kandel R, *et al*. Susceptibility to radiation-carcinogenesis and accumulation of chromosomal breakage in p53 deficient mice. Oncogene 1994; 9: 3731-6.

Lee96 van Leeuwen CJ, Rasmussen FB, Feijtel TCJ, *et al*. Risk assessment and management of new and existing chemicals. Environ Toxicol Pharmacol 1996; 2: 243-99.

Ler96 Leroux BG, Leisenring WM, Moolgavkar SH *et al.* A biologically- based dose-response model for developmental toxicology. Risk Anal 1996; 16: 449-58.

Lip98 Lipscomb JC, Fisher JW, Confer PD *et al*. In vitro to in vivo extrapolation for trichloroethylene metabolism in humans. Toxicol Appl Pharmacol 1998; 152: 376-87.

Loh99 Lohman PHM. Qualitative and quantitative procedures for health risk assessment. Mutat Res 1999; 428: 237-54.

Lov96 Loveless SE, Ladics GS, Gerberick GF, *et al*. Further evaluation of the local lynmph node assay in the final phase of an international collaborative trial. Toxicology 1996; 108: 141-52.

Mag69 Magnusson B, Kligman AM. The identification of contact allergens by animal assays. The guinea pig maximization test. J Invest Dermatol 1969; 52: 268.

Man96 Manjanatha MG, Shelton A, Aidoo A, *et al*. Comparison of in vivo mutagenesis in the endogenous Hprt gene and the lacI transgene of Big Blue rats treated with 7,12-dimethylbenz[a]anthracene. Mutat Res 1998; 401: 165-78.

Med94 Medinsky MA, *et al*. In vivo metabolism of butadiene by mice and rat: A comparison of physiological model predictions and experimental data. Carcinogenesis 1994; 15: 1329-40.

Mil97 Miller B, Albertini S, Locher F, *et al*. Comparative evaluation of the in vitro micronucleus test and in vivo chromosome aberration test: industrial experience. Mutat Res 1997; 392: 45-59.

Mil01 Mileson BE, Ferenc SA. Methods to identify and characterize developmental neurotoxicity for human health risk assessment: overview. Environ Health Perspect 2001; 109: 77-8.

Moo99 Moolgavkar S, Krewski D, Zeise L , *et al*., red. Quantitative Estimation and Prediction of Human Cancer Risks. Lyon: IARC, 1999.

Mul99 Müller K, Kikuchi Y, Probst G, *et al.* ICH-harmonised guidances on genotoxicity testing of pharmaceuticals: evolution, reasoning and impact. Mutat Res 1999; 436 : 195-225.

Mun98 Munro IC, Shubik P, Hall R. Principles for the safety evaluation of flavouring substances. Fd Chem Toxic 1998; 36: 529-40.

Mun99 Munro IC, Kennepohl E, Kroes R. A procedure for the safety evaluation of flavouring substances. Fd Chem Toxic 1999; 37: 207-32.

Noh96 Nohmi T, Katoh M, Suzuki H, *et al*. A new transgenic mouse mutagenesis test system using Spi and 6-thioguanine selections. Environ Mol Mutagen 1996; 28: 465-70.

NRC00 National Research Council. Scientific Frontiers in Developmental Toxicology and Risk Assessment. Washington: National Academy Press, 2000.

NRC92 National Research Council. Environmental Neurotoxicology. Washington: National Academy Press, 1992.

Nuw99 Nuwaysir E, Bittner M, Trent J, *et al.* Microarrays and toxicology: the advent of toxicogenomics. Molecular Carcinogenesis 1999; 24: 153-9.

OECD81 Organization for Economic Co-operation and Development. Guidelines for testing of chemicals. Test guidelines Nos 401: Acute toxicity; 402: Acute dermal toxicity; 403: Acute inhalation toxicity. Parijs: OECD, 1981.

OECD87 Organization for Economic Co-operation and Development. Guidelines for testing of chemicals. No 401: Acute oral toxicity. Updated guideline. Parijs: OECD, 1987.

OECD92 Organization for Economic Co-operation and Development. Guidelines for testing of chemicals. No 420: Acute oral toxicity: fixed-dose method. Parijs: OECD, 1992.

OECD92a Organization for Economic Co-operation and Development. Guidelines for testing of chemicals. No 406: Skin sensitisation. Parijs: OECD, 1992.

OECD96 Organization for Economic Co-operation and Development. Guidelines for testing of chemicals. No 423: Acute oral toxicity: acute toxic class method. Parijs: OECD, 1996.

OECD00 Organization for Economic Co-operation and Development. Guidelines for testing of chemicals. No 425: Acute oral toxicity: modified up-and-down procedure (revised draft guideline). Parijs: OECD, 2000.

OECD00a Organization for Economic Co-operation and Development. Guidelines for testing of chemicals. No 429: Skin sensitisation: local lymph node assay (draft new guideline). Parijs: OECD, 2000.

Ost83 Osterman-Golkar S, Farmer PB, Segerbäck D, *et al*. Dosimetry of ethylene oxide in the rat by quantitation of alkylated histidine in hemoglobin. Teratogen Carcin Mut 1983: 3: 395-405.

OTA90 Office of Technology Assessment. Neurotoxicity. Washington: OTA, 1990.

Par98 Parchment RE. Alternative testing systems for evaluating noncarcinogenic, hematologic toxicity. Environ Health Perspect 1998; 106: 541-58.

Pit95 Pitot HC. The role of receptors in multistage carcinogenesis. Mutat Res 1995; 333: 3-14.

Pfa98 Pfaller W, Gsraunthaler G. Nephrotoxicity testing in vitro - what we know and what we need to know. Environment Health Perspect 1998; 106: 559-70.

Pre98 Preston RJ. New approaches in genetic toxicology and their possible applications to cancer risk assessment. CIIT activities 1998; 18, no 3.

Ram84 Ramsey JC, Anderson ME. A physiologically based description of the inhalation pharmacokinetics of styrene in rats and humans. Toxicol Appl Pharmacol 1984; 73: 159-75.

Ram96 Rambukkana A, Pistoor FHM, Bos JD, *et al*. Effects of contact allergens on human Langerhans cells in skin organ culture: migration, modulation of cell surface molecules and early expression of IL-1ß protein. Lab Invest 1996; 79: 422-36.

Rei88 Reitz RH, Mendrala AL, Park CN, *et al*. Incorporation of in vitro enzyme data into the physiologically based pharmacokinetic model for methylene chloride: implications to for risk assessment. Toxicol Lett 1988; 43: 97-116.

Rei90 Reitz RH, Mendrala AL, Corley RA, *et al*. Estimating the risk of liver cancer associated with human exposures to chloroform using physiologically based pharmacokinetic modelling. Toxicol Appl Pharmacol 1990; 105: 443-59.

Rid96 Ridings JE, Barratt MD, Cary R, *et al*. Computer prediction of possible toxic action from chemical structure; an update on the DEREK system. Toxicology 1996; 106: 267-79.

Rom94 Romani N, Grüner S, Brang D, *et al*. Proliferating dendritic cell progenitors in human blood. J Exp Medicine 1994; 180: 83-93.

Sch95 Schwartz M, Buchmann A, Stinchcombe S, *et al*. Role of receptors in human and rodent hepatocarcinogenesis. Mutat Res 1995; 333: 69-79 .

Sch98 Schwetz B, Gaylor D. Alternative tests: carcinogenesis as an example. Environ Health Perspect 1998; 106: 467-72.

Sei94 Seibert H, Gülden M, Voss JU. An in vitro toxicity testing strategy for the classification and labelling of chemicals according to their potential acute lethal potency. Toxicol in vitro 1994; 4: 847-50.

Sei96 Seibert H, Balls M, Fentem JH, *et al*. Acute toxicity testing in vitro and the classification and labelling of chemicals. ATLA 1996; 24: 499-510

Set92 Sette WF, MacPhail RC. Qualitative and quantitative issues in assessment of neurotoxic effects. In: Tilson HA, Mitchell CL, red. Neurotoxicology. New York: Raven Press, 1992: 345-61.

She00 Sheiner LB, Steimer JL. Pharmacokinetic/pharmacodynamic modelling in drug development. Annu Rev Pharmacol Toxicol 2000; 40: 67-95.

Sil94 Silbergeld EK, Davis DL. Role of biomarkers in identifying and understanding environmentally induced disease. Clin Chem 1994; 1363-7.

Sil96 de Silva O, Basketter DA, Barratt MD, *et al*. Alternative methods for skin sensitisation. The report and recommendations of ECVAM workshop 19. ATLA 1996; 24: 683-705.

Sit00a van Sittert NJ, Boogaard PJ, Natarajan AT, *et al*. Formation of DNA adducts and induction of mutagenic effects in rats following 4 weeks inhalation exposure to ethylene oxide as a basis for cancer risk assessment. Mutat Res 2000; 447: 27-48.

Sit00b van Sittert NJ, Megens HJJJ, Watson WP, *et al*. Biomarkers of exposure to 1,3-butadiene as a basis for cancer risk assessment. Toxicol Sci 2000; 56: 189-202.

Sob96 Sobotka TJ, Ekelman KB, Slikker W, *et al*. Food and Drug Administration *Proposed* Guidelines for neurotoxicological testing of food chemicals. Neurotoxicology 1996; 17: 825-36.

Spe80 Spencer PS, Schaumburg HH, red. Experimental and Clinical Neurotoxicology. Baltimore: William and Wilkins, 1980.

Spi94 Spielmann H, Lovell WW, Hölze E, *et al.* In vitro phototoxicity testing. ATLA 1994; 22: 314-48.

Spi98a Spielmann H, Bochkov NP, Costa L, *et al*. Alternative testing methodologies for organ toxicity. Environ Health Perspect 1998; 106: 427-40.

Spi98b Spielmann H. Reproduction and Development. Environ Health Perspect 1998; 106: 571-6.

Sto98 Stokes WS, Marafante E. Introduction and summary of the 13th meeting of the scientific group on methodologies for the safety evaluation of chemicals (SGOMSEC): Alternative testing methodologies. Environ Health Perspect 1998; 106: 405-12.

Str79 Strauss GH, Albertini RJ. Enumeration of 6-thioguanine-resistant peripheral blood lymphocytes in man as a potential test for somatic cell mutations arising in vivo. Mutat Res 1979; 61: 353-79.

Tat89 Tates AD, Bernini LF, Natarajan AT, *et al*. Detection of somatic mutants in man: hprt mutations in lymphocytes and hemoglobin mutations in erythrocytes. Mutat Res 1989; 213: 73-82.

Tat95 Tates AD, Boogaard PJ, Darroudi F, *et al*. Biological monitoring in industrial workers following accidental exposure to high concentrations of ethylene oxide. Mutat Res 1995; 329: 63-77.

Tat99 Tates AD, van Dam FJ, Natarajan AT, *et al*. Measurement of hprt mutations in splenic lymphocytes and haemoglobin adducts in erythrocytes of Lewis rats exposed to ethylene oxide. Mutat Res 1999: 431: 397-415.

Ten95 Tennant RW, French JE, Spalding JW. Identifying chemical carcinogens and assessing potential risk in short-term bioassays using transgenic mouse models. Environ Health Perspect 1995; 103: 942-50.

Ten98 Tennant RW. Evaluation and validation issues in the development of transgenic mouse carcinogenicity bioassays. Environ Health Perspect 1998; 106: 473-6.

Ten99 Tennant RW, Butterworth BE, Preston RJ. Genetically engineered cancer models: three viewpoints on issues from testing to risk assessment. CIIT activities 1999; 19, No 8.

Tic01 Tice RR, Agurell E, Anderson B, *et al*. The single cellgel/Comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ Molec Mutagen 2001, in druk.

Til92 Tilson HA, Mitchell CL, red. Neurotoxicology. New York: Raven Press, 1992.

Til00 Tilson HA. Neurotoxicology risk assessment guidelines: developmental neurotoxicology. Neurotoxicology 2000; 21: 189-94.

Vae98 Vaes WHJ, Ramos EU, Verhar HJM, *et al.* Acute toxicity of nonpolar versus polar narcosis: Is there a difference? Environ Toxicol Chem 1998; 17: 1380-4.

Ver92 Veronesi B. In vitro screening batteries for neurotoxicants. Neurotoxicology 1992; 13: 185-96.

Vri97 de Vries A, van Oostrom CT, Dortant PM, *et al*. Spontaneous liver tumours and benzo(a)pyrene-induced lymphomas in XPA-deficient mice. Mol Carcinog 1997; 19: 46-53.

VROM01 Ministerie van VROM. Strategienota Omgaan met Stoffen. Den Haag, 2001.

Wal98 Walum E. Acute oral toxicity. Environ Health Perspect 1998; 106: 497-504.

Wal99 Walker VE, Jones IM, Crippen TL, *et al*. Relationships between exposure, cell loss and proliferation, and manifestation of Hprt mutant T-cells following treatment of preweanling, weanling and adult mice with N-ethyl-N-nitrosourea. Mutat Res 1999; 431: 371-88.

Wel92 van Welie RTH, van Dijck RGJM, Vermeulen NPE, *et al*.. Mercapturic acids, protein adducts, and DNA adducts as biomarkers of electrophilic chemicals. Crit Rev Toxicol 1992; 22: 271-306.

Wol00 Wolf CR, Smith G, Smith RL. Pharmacogenetics. Br Med J 2000; 320: 987-90.

Wri95 Wright AS, Aston JP, van Sittert NJ, *et al.* Molecular approaches to assess cancer risks. In: Thomas H, Hess R, Waechter F, red. Toxicology of industrial compounds. London: Taylor & Francis, 1995: 185-200.

Yam97 Yamamoto S, Hayashi Y, Mitsumori K, *et al*. Rapid carcinogenicity testing system with transgenic mice harbouring human phenotype c-Ha-RAS gene. Lab Anim Sci 1997; 47: 121-6.

Zac98 Zacharewski T. Identification and assessment of endocrine disruptors: limitations of in vivo and in vitro assays. Environ Health Perspect 1998; 106: 577-82.

A Presentation of the problem

B The Committee

Annexes

Annex **A**

Presentation of the problem

On 29 August 1996, the Committee for the 'Derivation of recommended health values' published the advisory report on 'Toxicology-based recommended exposure limits'. In its presentation letter accompanying that advisory report, the President of the Health Council of the Netherlands wrote the following:

"In parts, the Committee considers further elaboration to be possible on the basis of current knowledge".

One of the topics that was considered for such further elaboration was:

"a method for drawing up an integral toxicity profile".

Annex **B**

The Committee

- Dr WRF Notten, *chair*
  toxicologist; TNO Prevention and Health, Leiden
- Dr WFJPM ten Berge
  toxicologist; DSM, Heerlen
- Dr BJ Blaauboer
  toxicologist; IRAS, University of Utrecht
- Prof. VJ Feron
  Emeritus Professor of biological toxicology; University of Utrecht
- Prof. PHM Lohman
  Professor of radiation genetics and chemical mutagenesis; University of Leiden
- Dr G de Mik
  toxicologist; National Institute of Public Health and the Environment, Bilthoven
- Prof. WF Passchier, *advisor*
  Health Council of the Netherlands, The Hague
- Dr MN Pieters, *advisor*
  National Institute of Public Health and the Environment, Bilthoven
- Dr GMH Swaen
  epidemiologist; University of Maastricht
- Dr RA Woutersen
  toxicologist/pathologist; TNO Nutrition, Zeist
- Dr JA van Zorge, *advisor*
  Ministry of Housing, Spatial Planning and the Environment,The Hague

- Dr EJ Schoten, *secretary*
  Health Council of the Netherlands, The Hague
- Dr PW van Vliet, *secretary*
  Health Council of the Netherlands, The Hague

Scientific support:
- Dr NJ van Sittert
  toxicologist

Administrative support:
- M Javanmardi

Lay-out:
- J van Kan and M Javanmardi