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Initial assessment of the hazards and risks of new chemicals to man and the environment

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CONTENTS

SUMMARY

This report describes the initial hazard and risk assessment process for new substances at the National Institute of Public Health and Environmental Protection (RIVM) in The Netherlands. This assessment pertains to both man and the environment and is performed within the framework of the European Community (EC) Directive 79/831/EEC and the ensuing Dutch Chemical Substances Act. The report is restricted to the assessment of substances on the basis of the tests required at a market or production volume up to 100 tonnes per year or 500 tonnes cumulative. It has been written as a reference guide for those involved in the hazard and risk assessment process at RIVM in the first place, but also for the risk managers responsible for the regulation and the overall dossier evaluation of new substances, and for others interested in this subject, e.g. industry, non-governmental organizations and the public at large.

Step by step the essential elements of the hazard and risk assessment process are discussed: organizational aspects, data requirements, the determination of the acceptability of the data received, the scientific evaluation of the test methods and results, the exposure assessment, the comparison of exposure and toxicity data, and the uncertainty analysis. The report also discusses requirements for further testing following the initial assessment and concludes with a summary of recent and relevant developments in the European Community within the field of the assessment of new substances.

SAMENVATTING

Dit rapport beschrijft het proces van de eerste beoordeling van de gevaren en risico's van nieuwe stoffen op het RIVM. Deze beoordeling betreft zowel het milieu als de mens en wordt uitgevoerd in het kader van de Europese Richtlijn 79/831/EEG en de hieruit voortvloeiende Nederlandse Wet Milieugevaarlijke Stoffen. Het rapport beperkt zich tot de beoordeling van stoffen op basis van de proeven die vereist zijn bij een produktie- of marktvolume tot 100 ton per jaar of 500 ton cumulatief. Het is in de eerste plaats bedoeld als een gids voor hen die op het RIVM betrokken zijn bij de beoordeling van stoffen, maar ook voor beleidsmedewerkers die belast zijn met de regulering en de algehele evaluatie van dossiers van nieuwe stoffen en voor overige geïnteresseerden, bijvoorbeeld bij de industrie, particuliere organisaties en het algemene publiek.

De belangrijkste onderdelen van het beoordelingsproces worden stap voor stap besproken: organisatorische aspecten, de eisen met betrekking tot de in te dienen gegevens, de bepaling van de ontvankelijkheid van de verstrekte gegevens, de blootstellingsanalyse, de vergelijking tussen blootstelling en toxiciteit en de onzekerheidsanalyse. Het rapport bespreekt ook de eisen voor nader onderzoek volgend op de eerste beoordeling en besluit met een samenvatting van recente en relevante ontwikkelingen in de Europese Gemeenschap op het terrein van de beoordeling van nieuwe stoffen.

1. INTRODUCTION

The Dutch Chemical Substances Act (Anonymous, 1985) came into force on 1 February 1986. The Act, and the ensuing Notification Order (Anonymous, 1986), incorporate into national legislation the regulations of the European Community (EC) premarketing notification directive for new substances (Directive 79/831/EEC; EC, 1979), commonly called the "sixth amendment". New substances are defined as those which were not on the market within the EC at any time within the 10 years prior to 18 September 1981 and which, therefore, do not appear in the European Inventory of Existing Commercial Chemical Substances (EINECS; EC, 1987). The Dutch Act also contains rules for new substances before manufacturing and for the so called existing substances, which appear in EINECS. In principle, all new substances, intended to be manufactured or marketed in The Netherlands, have to be notified to the Chemical Substance Bureau of the Ministry of Housing, Physical Planning, and Environment. The system of notification before marketing is harmonized within the EC: notification of a new substance in one member state of the EC means that the notifier, a single company, may market the substance in all twelve member states. The notification must be accompanied by base set information, including data on chemical identity, volume, application(s), physico-chemical properties, and the toxicity of the substance.

The EC Directive 79/831/EEC requires an evaluation of the potential hazards/risks of notified substances to man as well as the environment. In The Netherlands, the Chemical Substances Bureau is responsible for coordinating these activities. The National Institute of Public Health and Environmental Protection (Dutch acronym: RIVM) advises the Chemical Substances Bureau with respect to the potential hazards and risks of new substances to man and the environment. Within the Institute, this task is coordinated by the Toxicology Advisory Centre and has been described briefly by Roghair (1988a). Occupational hazard and risk assessments are prepared by the Netherlands Organization for Applied Scientific Research TNO.

The aim of this report is to describe in detail the hazard and risk assessment process for new substances at the RIVM. This description will be restricted to base set level, i.e. will consider the assessment of substances on the basis of those tests which are required at a market or production volume of up to 100 tonnes/year or 500 tonnes cumulative. However, test requirements for higher tonnage levels will be discussed. The report is not meant to be a "cookery book" for hazard and risk assessment, but primarily to be a reference guide for those involved in the hazard and risk assessment of new substances at the RIVM. It also intends to give the risk manager a detailed overview of the risk assessment process including a descrip-

tion of the possible conclusions and the uncertainties therein. And finally, the report will give other interested parties e.g. industry, non-governmental organizations and the public at large, sufficient understanding of the risk assessment process to appreciate its significance for risk management by the Dutch competent authorities.

This publication is based on the long-standing experience of experts at the RIVM in the area of hazard and risk assessment of chemical substances. Approximately 700 new chemicals with a market volume of over 1 tonne/year have been evaluated within the scope of the EC Directive 79/831/EEC in the period 1983-1991. In the same period, over 400 limited announcements (for substances with a market volume below 1 ton/year) have been processed. The report can, however, only reflect the current opinions of the experts involved and the current state of affairs vis-á-vis legal requirements. Therefore, in the rapidly developing area of hazard/risk assessment, regular updating is a necessity. It is the intention of the authors to distribute updates periodically. Aspects of the assessment at higher tonnage levels will be the subject of a future report.

Abbreviations and the most essential concepts which are used in this report are explained both in the text and in Annex I.

2. OVERVIEW OF THE EVALUATION PROCESS FOR NEW SUBSTANCES

2.1 The notification procedure

The general notification procedure for new substances within the scope of the EC Directive 79/831/EEC is outlined in figure 1. A company which intends to market a new substance in the European Community must notify the competent authority in one of the member-states at least 45 calendar days before it is actually marketed. The competent authority in The Netherlands is the Chemical Substances Bureau. Both the Ministry of Housing, Physical Planning and Environment, and the Ministry for Social Affairs and Employment take part in this Bureau. In addition to the premarketing notification, the Dutch Chemical Substances Act also requires notification prior to the production of a new substance in The Netherlands.

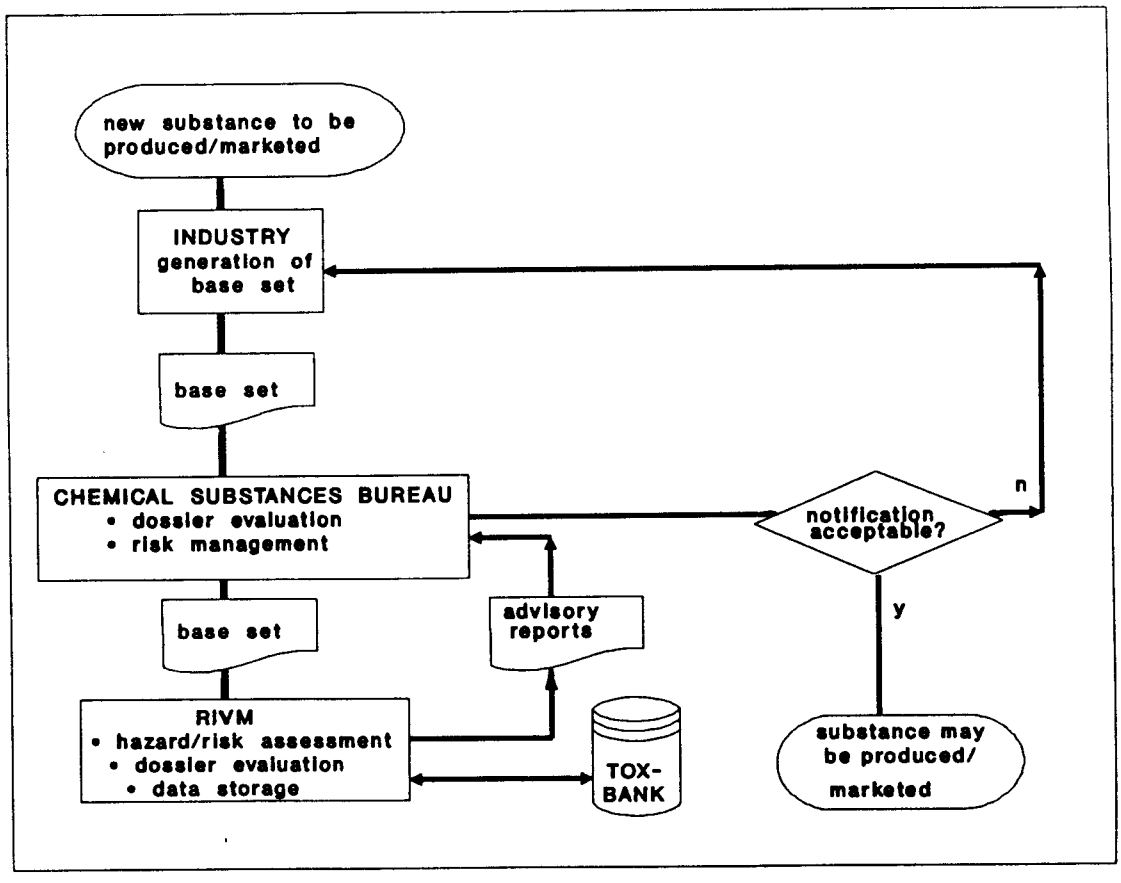

Fig.1: General notification procedure for new substances within the scope of EC Directive 79/831/EEC.

Notifications must be accompanied by a base set of information. The premarketing base set is shown in figure 2. The country which has received notification (lead country) immediately sends a summary notification to the European Commission for distribution to the other member-states. The premanufacturing notification (PMN) base set as required in The Netherlands is a subset of the EC base set: ecotoxicological data are not compulsory and the Dutch competent authority may rule that the subacute test can be dispensed with. Limited announcements, containing a very limited data set, apply to certain substances for research and development purposes and to substances which are produced, imported or marketed in quantities of less than one tonne per year in the European Community. These announcements have not yet been harmonized in the EC.

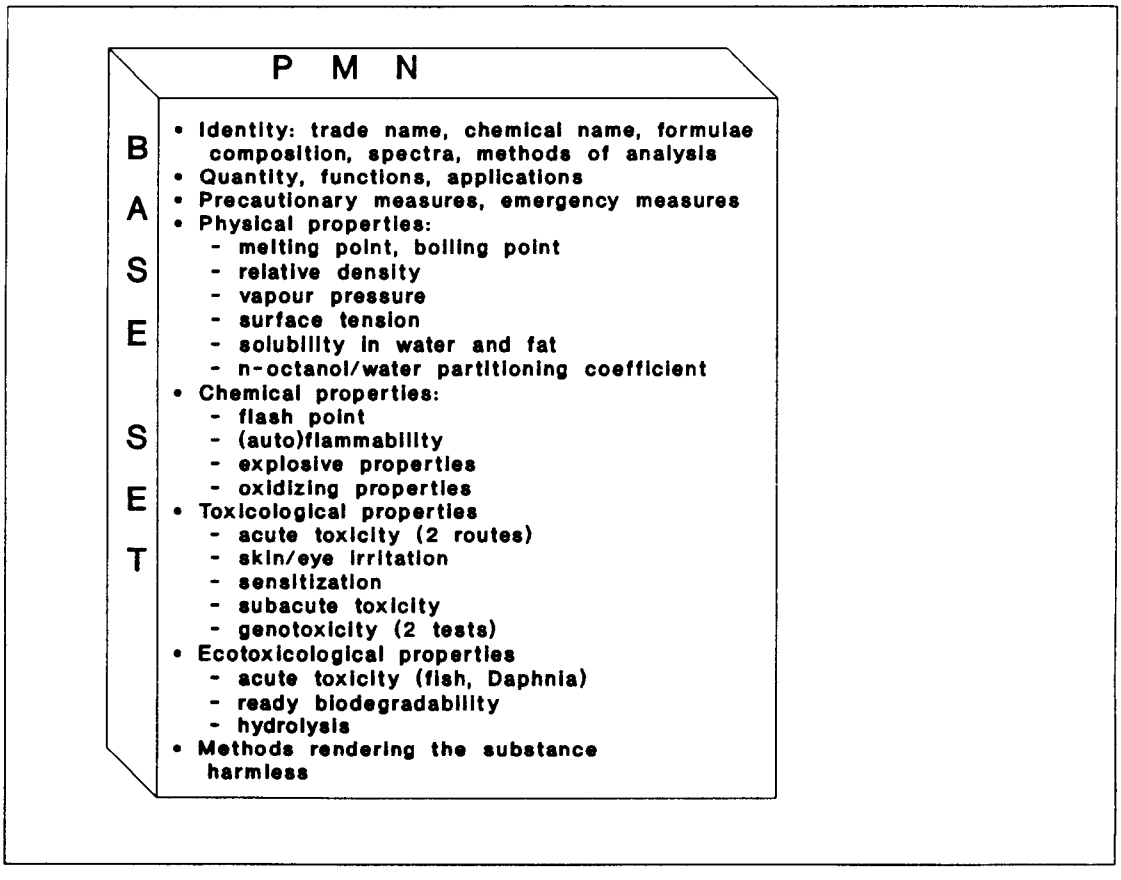

Fig.2: Premarketing base-set for new substances within the scope of EC Directive 79/831/EEC.

The Chemical Substances Bureau decides, based on expert advice, whether the submitted dossier is complete and whether the tests required have been carried out according to the guidelines (chapter 3). If the notification proves to be unacceptable, the notifier receives notice within 30 days. The Bureau is also responsible for risk management and for further testing

requirements to the notifier (chapter 7) with respect to all notified new substances irrespective of the country of first notification. In addition, the Directorate General of Labour of the Ministry for Social Affairs and Employment is responsible for matters relating to occupational hygiene, and the Ministry of Welfare, Health and Cultural Affairs for matters relating to product policy. The risk management options available to the Administration are prescribed by the Chemical Substances Act (sections 24-40; Anonymous, 1985) and summarized elsewhere (MHPPE, 1987). Following the 45 day period the new substance has access to the EC market (premarketing notifications) or may be manufactured in The Netherlands (premanufacturing notifications) provided that:

- no immediate legal measures have been taken to ban import or production in case of unacceptable risks to man and/or environment,
- the notification and the tests were in accordance with the legislative regulations,
- the substance or preparation is packaged and labelled in accordance with the rules.

The dossier evaluation and risk management by the Chemical Substances Bureau is based on scientific advice from the National Institute of Public Health and Environmental Protection (RIVM) as far as the public at large and the environment are concerned. Occupational hazard and risk assessments are prepared by The Netherlands Organization for Applied Scientific Research TNO. At the RIVM, all data submitted with the notifications and all hazard and risk assessments are stored in a data bank called TOXBANK (Bourgeois & Heijna-Merkus, 1987; Hobo, 1990). This data bank currently is being converted to a relational data bank called TOXIS in order to improve retrieval and research capabilities (Audit, 1990).

2.2 RIVM tasks

The tasks of the RIVM regarding the evaluation of new substances, and the main procedures and responsibilities within the Institute are shown in figure 3. Some aspects have already been described earlier (Roghair, 1988a). The evaluation procedures at the RIVM are coordinated by the Toxicology Advisory Centre (TAC), which is in frequent contact with the Chemical Substances Bureau (CSB). As indicated in figure 3, the TAC exploits the expert knowledge available at the Institute in order to maintain and increase the quality of the advisory reports. At the various stages in the evaluation procedure experts are frequently consulted. In addition, a hazard and risk assessment procedure for new substances has been developed in cooperation with these experts, and is being developed further, to meet the demands of the CSB for a quick, reproducible, reliable and transparent assessment method (chapters 4-7).

As pointed out in section 2.1, base set data arrive at the RIVM via the CSB either as complete dossiers containing the original test reports in case of Dutch notifications, or as summaries.

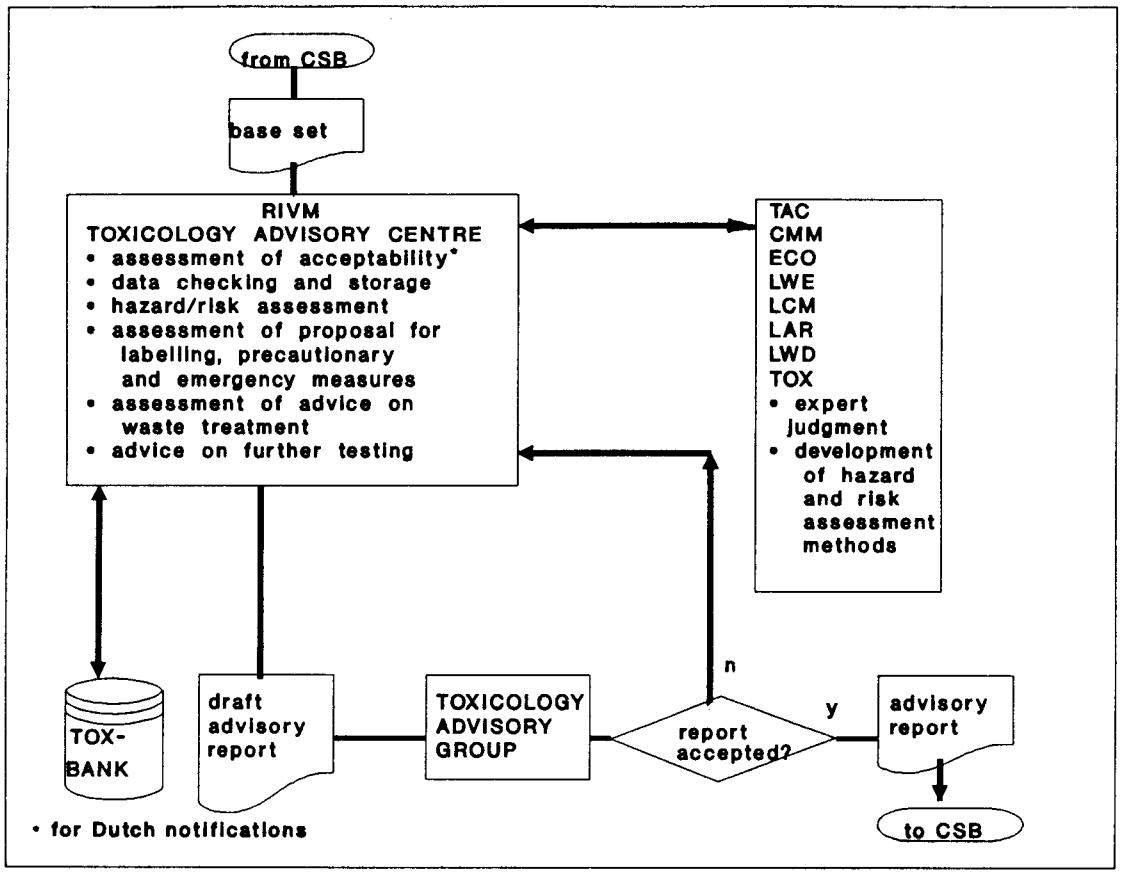

Fig.3: Advisory tasks of the RIVM towards the CSB within the scope of the Chemical Substances Act for new substances

Abbreviations:

RIVM = National Institute of Public Health and Environmental Protection
CSB = Chemical Substances Bureau
TAC = Toxicology Advisory Centre
CMM = Centre of Mathematical Methods
ECO = Laboratory of Ecology
LWE = Laboratory of Waste and Emissions
LCM = Laboratory of Carcinogenesis and Mutagenesis
LAR = Laboratory of Air Research
LWD = Laboratory of Water and Drinking Water Research
TOX = Laboratory of Toxicology

Limited announcements may contain one or more original reports. Written advice on the acceptability of Dutch notifications has to be sent to the CSB within 15 calender days from the date of receipt at the TAC (chapter 3).

The data from accepted complete dossiers and summaries are evaluated further and stored into the databank as shown in figure 3. A draft advisory report is submitted to the Toxicology Advisory Group for final review. The Toxicology Advisory Group is composed of RIVM experts in the areas of emissions and waste treatment, physico-chemical properties, environmental fate, human toxicology, ecotoxicology, and hazard and risk assessment. Once approved, the final advisory report, retrieved from TOXBANK and containing the data in a fixed format, an evaluation of these data, questions to the notifier, and a hazard and risk assessment, is sent to the CSB, the Directorate General of Labour, and the Ministry of Welfare, Health and Cultural Affairs within 30 days from the date of receipt at the TAC.

A summary of the time schedule as fixed for the evaluation procedure of new substances in The Netherlands is shown in table 1.

Table 1: Deadlines in the evaluation procedure

Activity	Deadline (calender days)	
	CSB	RIVM
Assessment of acceptability	30	15
Hazard/risk assessment	45	30
Emergency action	45	

In addition to these reports, the RIVM advises the governmental departments mentioned before (section 2.1) on further testing requirements (chapter 7), test methods (chapter 4), and general aspects relating to the human and environmental assessment of new substances (chapter 4-6). Experts from the RIVM also participate in international expert groups working in these fields (chapter 8), especially those of the European Community and of the Organization for Economic Cooperation and Development (OECD).

2.3 Hazard and risk assessment, concepts

For a proper understanding of the chapters that follow, the conceptual framework with respect to the assessment of substances has to be outlined first. To this effect, reference is made to a

publication of the US National Research Council (NRC, 1983) and one of the European Community (EC, 1990d). Figure 4 shows the basic elements of the assessment of chemical substances. In this report hazard is defined as the potential of a substance to cause adverse effects at a particular degree of exposure. Risk is defined as the probability of a substance to cause adverse effects.

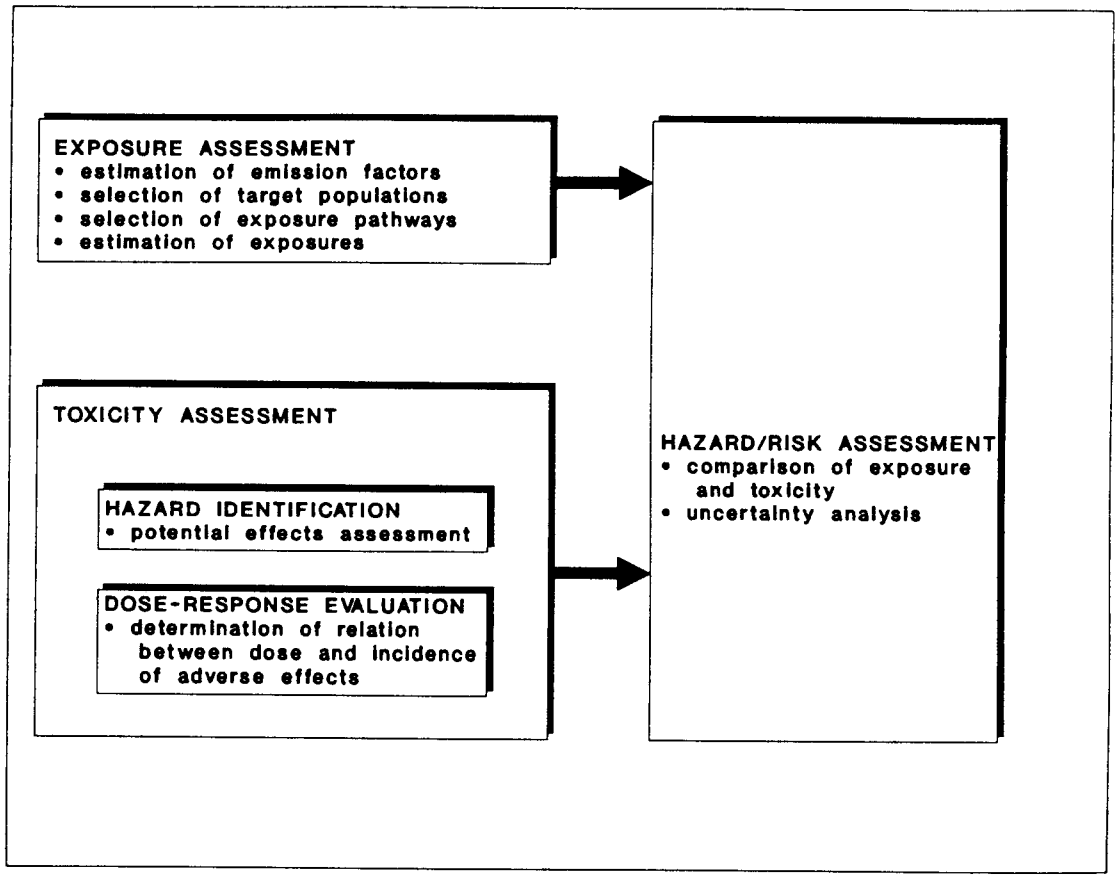

Fig.4: Concepts of hazard and risk assessment

Exposure assessment for new substances involves an estimation of the emission rate of a substance, a selection of the target populations and exposure pathways, and an estimation of point concentrations for specific pathways, based on predictive modelling results. The toxicity assessment includes two components: hazard identification and dose-response assessment. Hazard identification is the process of determining whether exposure to a substance can cause an increase in the incidence of an adverse effect. One of the outcomes of this stage is the classification and labelling of the substance. The dose-response evaluation involves establishing the relationship between the dose and the incidence of adverse effects in a particular group of test organisms and, through extrapolation, in an entire population.

In hazard assessment, the results of the exposure assessment and the toxicity assessment are

combined to establish the potential to cause adverse effects qualitatively or quantitatively e.g. by using hazard indices such as the PEC/NEC-ratio (Predicted Environmental Concentration/No-Effect Concentration) and the DWE/DI-ratio (Dose Without Effect/Daily Intake). In risk assessment, the relation between the predicted exposure and adverse effects is expressed using probability functions.

3. AVAILABILITY AND ACCEPTABILITY OF DATA

3.1 Availability of data

Anyone giving notification of the import or commercialization of a new substance in the Netherlands must provide the base set of data (Fig.2), a statement on the expected adverse effects of the substance, and a proposal for classification and labelling. Premanufacturing notifications do not require ecotoxicological data and the Directorate-General of Labour of the Ministry of Employment and Social Security decides on the necessity of a subacute test in a mammalian species. According to the Notification Order (Anonymous, 1986) dispensation from the subacute test can be given:

- on the basis of existing toxicological information on substances with an identical chemical structure,
- in case of negligible exposure of workers,
- in case of the absence of repeated exposure of workers.

However, submission of the data omitted from the base set in a premanufacturing notification may be required at any later date if deemed necessary.

Certain data need not be submitted with both types of notification for technical or scientific reasons which must be explicitly explained by the notifier. Some of these cases will be discussed below.

Polymers

> A polymer means a substance consisting of molecules characterized by the sequence of one or more types of monomer units and comprising a simple weight majority of molecules containing at least 3 monomer units which are covalently bound to at least 1 other monomer unit or other reactant and consists of less than a simple weight majority of molecules of the same molecular weight. Such molecules must be distributed over a range of molecular weights wherein differences in the molecular weight are primarily attributable to differences in the number of monomer units. In the context of this definition a "monomer unit" means the reacted form of a monomer in a polymer
>
> EC, 1990a

Fig.5: Definition of a polymer

Directive 79/831/EEC provides that "polymerizates, polycondensates, and polyadducts, except those containing in combined form 2% or more of any monomer unmarketed before 18 September 1981 are considered to have been notified". Subsequently it was proposed to adopt

a new definition of polymers (Fig. 5) and to divide the polymers into polymers of low concern and polymers of high concern (EC, 1990a). The latter group requires the complete base set and, in addition, the number average molecular weight, the molecular weight distribution, impurities, identity and concentration of starting materials, end groups, and reactive groups. Notification of polymers of low concern (number average molecular weight > 10,000, less than 1% with a molecular weight < 1000, extractivity in water at pH 7 < 1 mg/l) requires a specific testing package including the base set data on identity as specified above for polymers of high concern, most of the base set physico-chemical tests, determination of the particle size, elution tests and tests on thermal stability, but in principle no (eco)toxicological tests. Testing procedures have also been proposed (EC, 1991a).

Physico-chemical properties

- The determination of the boiling point is not necessary for substances with a melting point well above room temperature (>50 °C) and for substances which decompose below the boiling point.
- The determination of the vapour pressure of substances with a high melting point often presents problems and may be omitted.
- The determination of the surface tension is impossible for solid substances with low water solubility, i.e. lower than 0.01 mg/l.
- The octanol-water partitioning coefficient cannot be determined for substances with low solubility in either water or octanol. Surface active substances may also present problems. The determination is not sensible for substances that dissociate.
- The flashpoint need not be determined for solids.
- The test for explosive properties is irrelevant if the available thermodynamic information (heat of formation, heat of decomposition, absence of certain reactive groups in the formula) establishes beyond reasonable doubt that the substance is incapable of decomposing, forming gases and releasing heat very rapidly (for examples, see EC, 1985).
- The test for oxidizing properties need not be performed if the chemical structure indicates that the substance cannot have oxidizing properties. The test is not applicable to liquids, gases, explosive or highly flammable solids, organic peroxides and combustible substances that melt under the conditions of the test. If a combustible substance melts, its mixture with cellulose may burn more rapidly than the reference mixture because the melting substance will spread over the large surface of the cellulose. A "false positive" result is then obtained. This behaviour of increased flammability can only be confirmed by performing the test in a nitrogen atmosphere or by mixing the substance with an inert substance such as kieselguhr instead of cellulose.

Toxicological properties

- The investigation of the acute toxicity of substances other than gases should take place via two routes at least, one of which should be the oral route. Gases and volatile liquids should be tested by inhalation. The need for acute inhalation testing has to be considered on the basis of all available data regarding substance properties (particle size, vapour pressure, volatility, Henry coefficient, chemical structure, toxicological profile) and exposure (possibility of the formation of dust, aerosol, vapour/gas). An acute inhalation study cannot be performed on solids that do not sublime or form respirable particles and on non-volatile liquids that do not produce aerosols. A dermal test is pointless if it is known that the substance does not penetrate the skin.

  A special EC Working Group is preparing a strategy document, which will act as a guideline for choosing appropriate administration routes in base set and post base set testing (EC, 1991b).
- Skin and eye irritation tests should not be submitted for substances that react strongly acid (pH < 2) or alkaline (pH > 11.5) and can be expected to be corrosive. If convincing evidence of severe effects is available from well validated in vitro or in in vivo tests, complete irritation tests also may not be required. In any case, eye irritation testing should be avoided when a substance has been shown to be corrosive in the dermal irritation test (EC, 1989a).
- A substance may be notified as a skin sensitizing agent without further testing if it has a chemical structure which is closely related to that of a known skin sensitizing agent or contains an impurity which is a known sensitizing agent.
- The route of administration in the 28-day test should be the most appropriate with regard to the intended use, the acute toxicity and the physical and chemical properties of the substance. In the absence of contra-indications, the oral route is the preferred route of exposure in order to gain insight into the potential of the substance to cause systemic toxicity.

  A special EC Working Group is preparing a strategy document, which will be a guideline to choose appropriate administration routes in base set and post base set testing (EC, 1991b).

Ecotoxicological properties

- If ready biodegradability is not expected of a substance, e.g. substances such as textile dyes required to be stable during use, the notifier may notify the substance as a not-readily biodegradable product without performing a ready biodegradability test and instead submit an inherent biodegradability test immediately (EC, 1986c). A biodegradability test is meaningless for a substance which rapidly reacts with water to form products with known environmental behaviour.
- The hydrolysis test need not be performed for substances without hydrolysable groups (see

section 4.4.1.1) nor for readily biodegradable substances. Not performing a hydrolysis test merely because of "poor solubility" cannot be accepted unless it is convincingly made clear that the substance or its products cannot be measured in water. For example, it may be possible to measure the hydrolysis rate of a poorly soluble substance by quantifying the soluble hydrolysis products formed with a sum parameter such as DOC (dissolved organic carbon).

3.2 Acceptability of data

3.2.1 GLP and QA

The tests for a base set must be carried out in accordance with the principles of Good Laboratory Practice (GLP) (OECD, 1982; Strik, 1988). Good Laboratory Practice guidelines describe how a laboratory should work, how it should be organized and how it can produce valid data. For example, a laboratory should have written standard operating procedures (SOPs) relating to all the important aspects of the laboratory operations and stipulating exactly which data must be registered, how reporting of data must be carried out and which requirements must be met by the laboratory and its staff. The guidelines should be implemented by a Quality Assurance program, i.e. an internal control system designed to ascertain that tests are in compliance with GLP principles. Laboratory inspections and study audits are conducted on a regular and routine basis by competent GLP Inspectors to determine the degree of conformity of laboratories and laboratory studies with GLP principles and to determine the integrity of data. The Notification Order (Anonymous, 1986) allows the possibility for certain tests, e.g. the determination of physico-chemical properties, to be excluded from this requirement.

The report should be signed and dated by the study director and should include names, dates, and signatures of the principal scientists, a Quality Assurance statement certifying the dates of inspection and the dates any findings were reported to the management and study director, and a statement, signed and dated by the study director, indicating acceptance of responsibility for the validity of the study and confirming that the study was conducted in accordance with GLP principles. It is important that the test and reference substances are carefully identified by name, code, purity, and composition or other appropriate characteristics. The stability and homogeneity of the test substance under the conditions of the test should be described.

3.2.2 Test guidelines

The tests for a base set must also be carried out in accordance with internationally recognised

test guidelines, preferably those of the EC, the so called Annex V methods (EC, 1984) and the OECD (1981). Other guidelines may be accepted if they are essentially in conformity with the above mentioned guidelines and if the notifier satisfactorily justifies any deviation.

The Annex V base set test guidelines for toxicology of the EC are currently being updated. The methods have already been accepted by the National Experts for Toxicology and Mutagenicity Methods (EC, 1989a). Several OECD guidelines are also being updated or have recently been updated. Updating of EC and OECD guidelines is a continuous process. Formal adoption often takes a considerable length of time. In general, revised methods, once approved by the national experts, are accepted for evaluation and are even preferred over the original methods. Formal adoption is no prerequisite to apply updated test protocols or to conduct new or modified test methods.

For further discussions on test guidelines: see chapter 4.

Guidance notes for completing a summary notification dossier have been issued (EC, 1989c). These notes contain further data on the type of information required.

4. EVALUATION OF BASE SET DATA

4.1 Evaluation of data regarding identity

4.1.1. Methods

The identity of the substance is disclosed by its IUPAC, trade, and other names, CAS number, empirical and structural formula, composition, and spectral data. The spectral analyses must be performed on the substance as marketed; in case of interference by impurities, the spectrum must also be taken on the purified substance. Methodical details have been described (EC, 1986b)

4.1.2 Results

The evaluation of data regarding the chemical identity of notified substances mainly deals with comparing the chemical name of each of the distinct compounds to its molecular structure, empirical formula and molecular weight. To this end, use is made of the recommendations given by IUPAC on the nomenclature of both organic and inorganic compounds (IUPAC, 1979; IUPAC, 1980).

In addition to the compounds forming the bulk of the notified substance, the identity of the main impurities is also evaluated. Spectra may help to check the consistency of the reported structure and to identify any impurities not reported through unassigned peaks. In case of renotification, spectra should be compared to establish whether the substances are identical. To be able to do this, the spectra should be of good quality with readable abscissa and ordinate. Changes of the scale should be clearly indicated as well as any amplifications or changes of the base line. The spectra must include the name of the substance, the purity of the sample, the type and name of the spectrometer used, the operating temperature, the name of the solvent (spectroscopically pure) and the concentration of the solution, the name of the reference solvent(s), the name of the performing laboratory and the name of the head of the laboratory (EC, 1986b).

4.2 Evaluation of physico-chemical data

4.2.1. Methods

The physico-chemical data are determined according to the OECD guidelines for testing of

chemicals (OECD, 1981) or according to Annex V of the EC directive (EC, 1984).

Chessels et al. (1991) reviewed experimental methods used for measuring the octanol-water partition coefficient (K_{ow}). A comparison is also made between measured values and values calculated. It is shown that the difference between individual experimental methods being used is small for compounds having a log K_{ow}-value below 5.5, whereas for these compounds there is also little difference between calculated and measured Kow-values. Major differences are found for compounds having log K_{ow}-values over 5.5. Calculation for these compounds usually gives an overestimation (up to a factor of 10) of the K_{ow}.

4.2.2 Results

The main problems encountered in the determination of physico-chemical properties generally relate to one of the following categories:

1 - The physical state of the notified substance (solid, liquid or gas).

2 - The limited solubility in either water of octanol.

3 - Problems related to notified substances being mixtures of varying composition.

4 - The instability of the notified substance, usually upon contact with water.

Ad 1: Depending on the physical state of the substance and its stability at elevated temperatures, decomposition of the compound is often observed during the determination of the melting point and/or the boiling point. Additionally, for compounds having a high boiling point problems are often encountered regarding the determination of the vapour pressure. Some substances contain volatile impurities, which will result in a unrealistically high vapour pressure. It is recommended to try to remove these impurities before measuring the vapour pressure.

Ad 2: For compounds having a very low solubility in either water or octanol it often is impossible to determine the surface tension and the octanol-water partition coefficient (K_{ow}). In the final risk assessment these properties are important since they are used as key parameters in assessing the predicted environmental concentrations (PEC). In case no surface tension could be measured it is assumed that the notified substance does not lower the surface tension, indicating that additional sorption to surfaces (sediment-water, soil-water, soil-air, water-air) is not expected to occur in the environment. In case it has been impossible to experimentally determine the octanol-water partition coefficient, in general two distinct approaches are possible:

a. For compounds having a solubility below the detection limit in either of the two phases of the octanol-water system, yet having a solubility in the distinctly pure phases above the

detection limit, as a first estimate the octanol-water partition coefficient is calculated by dividing the solubility in octanol by the water-solubility.

b. For compounds for which the solubility is below the detection limit in all cases, the octanol-water partition coefficient is calculated according to the procedures given by Leo & Hansch and as implemented in the computerized ClogP3 algorithm by Leo & Weininger (1989). It should be noted that the method of Leo & Hansch is only applicable to non-ionized organic compounds having a log K_{ow}-value below 6.

Ad 3: The EC directive requires that the measurements have to be carried out on the substance as marketed. The main problems related to notified substances being mixtures of varying composition deal with the fact that the physical properties of the bulk only give limited information on the properties of the distinct compounds. This is especially important to the vapour pressure and the octanol-water partition coefficient of the distinct compounds, which may differ significantly from the 'bulk'-values measured; especially in the case of vapour pressure, the value reported may mainly be due to the presence of a limited number of low-boiling compounds.

At the moment no solutions are available for the problems mentioned. It could be suggested that in the case of mixtures, apart from measuring the physical-chemical properties of the bulk, also the physical-chemical properties of the at least partially purified distinct compounds should be measured. Obviously this is only applicable to those mixtures for which, from a physical-chemical point of view, purification is possible.

Ad 4: For hydrolytically instable substances it often is impossible to measure the K_{ow}. For these compounds too, the octanol-water partition coefficient can usually be calculated according to the procedures given by Leo and Hansch and as implemented in the computerized ClogP3 algorithm by Leo & Weininger (1989).

4.3 Evaluation of (eco)toxicological data

4.3.1 Acute mammalian, oral, dermal, inhalation toxicity

4.3.1.1 *Methods*

Limit tests should always be considered before carrying out any full acute toxicity tests in order to avoid unnecessary suffering of experimental animals. For the oral route, a new test based on toxicity rather than mortality, the Fixed-Dose Procedure (van den Heuvel, 1990), is accepted

and recommended as an alternative to the OECD 401 and EC Annex V.B1 LD_{50} test. This test was approved for inclusion in the EC base set by the EC National Experts for Toxicology Methods (EC, 1990b). This test avoids death of animals as an end-point, and instead relies on the observation of clear signs of toxicity developed at one of a series of fixed dose levels.

At present, non-animal tests for acute toxicity cannot fully replace in vivo tests. This conclusion was recently reached in a seminar sponsored by the European Commission (EC, 1989b)

4.3.1.2 *Results*

If properly conducted, an acute test may provide data on signs of intoxication, time to onset, duration, and reversibility of toxic effects, dose-response relationships, sex-specific effects, organs, tissues and functions affected, and mode of action. Evidently, the test is more than a lethality test purely for the purpose of classification and labelling. It should also be borne in mind that the precision and accuracy of LD_{50} values are limited. Typical 95% confidence bounds obtained in traditional single dose tests can extend over factors ranging from approximately 1 to 4 (Enslein et al., 1989). As for accuracy: in two replication studies under the auspices of the European Commission, in which a total of 100 laboratories in 13 countries participated, maximum to minimum ratios ranged from 2.84 to 11.89 in the first study and, using a more specified protocol, from 2.44 to 8.38 in the second one (Enslein et al., 1989).

The slope of the dose response curve can give insight into toxicokinetic characteristics. A steep slope, for example, may indicate rapid absorption and/or rapid onset of action. A flat slope indicates a large margin of safety between the lethal dose and the lowest-effect-dose.

4.3.2 Primary irritation: skin and eye irritation

4.3.2.1 *Methods skin irritation tests*

The main purpose of primary irritation tests is to gain information on the potentially deleterious effects of substances and preparations which are likely to be met in the normal use of these substances. This information, i.e. occurrence and severity of the effects, is utilized to classify hazard. It has been suggested that the dermal route of exposure to general chemicals and pesticides is the most significant one in and around the house and at work (NIOSH, 1980). Primary irritation may be defined as a local, reversible, inflammatory response of normal living skin to direct injury caused by a single application of a chemical agent without the involvement of an immunological mechanism.

Both the OECD and the EC have published a guideline for acute dermal irritation testing (OECD, 1981, guidelines 404, and EC, 1984 and 1989a, guideline Annex V.B4). The guidelines appear to be technically straightforward. However, there are several pitfalls which may distort the results and may lead to interlaboratory variations

The primary dermal irritation test is basically similar to the test introduced by Draize et al. in 1944. The major changes are the reduction of the application period from 24 to 4 hours and that the test substance is only applied on intact skin. The application on abraded skin has been omitted because the results did not add any information to the results obtained from the intact skin and for animal welfare reasons (Nixon et al., 1975; McCreesh & Steinberg, 1977). The test substance is applied to the skin of, in general 3 or more, animals in either a dose of 0.5 ml of a liquid, the liquid usually being undiluted, or 0.5 g of a sufficiently moistened solid, which may be pulverized if this is necessary to assure good contact with the skin.

The test substance should be covered with a gauze patch, which should be loosely held in contact with the skin by a semi-occlusive bandage. The application method introduces a number of factors which increase interlaboratory variations. Particularly the degree of occlusiveness of the bandages can considerably influence the severity of the skin irritation.

The albino rabbit is the preferred test animal species. However, other mammalian species are allowed. Many studies with different animal species are performed to compare the ability to predict human responses. An incomplete evaluation shows the following array of species with increasing susceptibility to irritation: man - dog - rat - hairless mouse - guinea pig - rabbit (Davies et al., 1972; Kaestner, 1977; MacMillan et al., 1975; Nixon et al., 1975; Roudabush et al., 1965). There is a continuous debate whether the rabbit should be the preferred animal species because of its particular susceptibility to irritation. A major benefit of the rabbit model is its greater ability to compare substances in the weak to mild-moderate range of irritancy. This may lead to a more accurate classification and provide a safety factor, because of a margin between the occurrence of irritancy in the rabbit and the likelihood of irritation in man.

Irritation is broadly specified by the occurrence of a significant inflammation being present at 24 hours or more after the end of the exposure period and characterized by erythema and oedema. It has to be mentioned that this definition is purely descriptive and does not define the underlying mechanism. The various grades of erythema and oedema are recorded by a standard numerical system.

A task group of experts of the European Chemical Industry Ecology and Toxicology Centre has

recently reviewed the OECD and EC guidelines (ECETOC, 1985).

4.3.2.2 *Results skin irritation tests*

A chemical is considered to be irritant, if after patch removal signs of inflammation can be detected. This inflammation is significant, if the mean value of the scores, denoted as primary irritation index, is two or more (on a scale of 0 to 4) for either erythema and eschar formation or oedema formation (EC, 1979). If only three animals are used, the phenomena have to be scored for each animal separately and a score of two or more has to be observed in at least two animals. Skin reactions can occur in various gradations. In the scoring system according to Draize et al. (1944) this is acknowledged and chemicals are classified into four classes: not, slightly, moderately and severely irritating, respectively. However, the EC classification is restricted to irritant or non-irritant.

In general, irritation is based on the subjective evaluation of erythema and oedema. However, more signs of irritation, like scab formation, hyperplasia, desquamation, alopecia, surface fissuring, discolouration, etc. can be recorded. Although these effects should be fully described, there is no advice in the EC method on the use of these additional signs of irritation with respect to classification. It is our opinion that these effects should be considered as unwanted. Therefore, a chemical inducing such effects may be classified as an irritant.

As stated before erythema and oedema should persist at least 24 hours, but another prerequisite is that these phenomena do occur within the time frame of 72 hours after patch removal, because the observations at 24, 48 and 72 hours after patch removal have to be used for the calculation of the mean value over all the animals. The study of Martens et al. (1987) suggests that these observation times may not coincide with the summit of the erythema reaction. The mean time of the peak reactions of 30 compounds, tested according to the OECD-guideline, was 88 hours (range 37-140 hours) after application. Evaluated according to the EC-directive, 25% of the compounds tested showed a mean score for erythema < 2, although they showed periods longer than 24 hours with a mean score > 2. It is suggested that the early formation of erythema and oedema is related to the irritating properties of the substances and that the occurrence of late stadia is a reflection of increased cellular metabolism related to healing induced by infiltrating leucocytes releasing vasoactive mediators.

According to the EC guidelines substances or preparations are considered to be corrosive if they produce full thickness destruction of skin tissue in at least one animal during the test for skin irritation. In our opinion more effects indicate the corrosiveness of chemicals. For instance,

skin irritation. In our opinion more effects indicate the corrosiveness of chemicals. For instance, tissue destruction, or to a lesser extent necrosis, can also cause scarring. Furthermore, all skin effects which do not show reversibility within fourteen days - in this period the rabbit skin fully regenerates - have to be considered permanent alterations of the normal skin. These effects are unwanted and therefore these substances should be labelled as corrosive. Substances of extreme pH (pH $\leq$ 2 or pH $\geq$ 11.5) need not be tested in animals because it is assumed that they induce severe skin irritation or corrosion. Young et al. (1988) indicated that pH alone does not accurately predict the effects and that the buffer capacity of the substance should also be considered.

The research for alternatives for animal skin testing is enormous. This is partly due to the large distress with may accompany the test, but also to the apparently straightforward nature of the skin reactions. A large number of *in vitro* models are developed, varying from cell cultures to tissue cultures and artificial skin. At the moment several validation studies are in progress. Main topic in these studies is the correlation of the *in vitro* data with the *in vivo* data. Previous studies (ECETOC, 1990; EC, 1990c) have shown that a good correlation can be obtained with several *in vitro* methods. For that reason *in vitro* methods are used in prescreening studies. For the introduction of *in vitro* methods in regulatory skin irritancy testing, not only a good *in vitro* - *in vivo* correlation is of importance, but also the acceptance of a certain amount of risk. In the OECD the debate on incorporating *in vitro* methods has just started.

4.3.2.3 *Methods eye irritation tests*

Besides the dermis much attention is paid in primary irritation tests to the eye. Not only the incidence of accidental eye injuries but also ethical reasons, like the prevention of loss of sight or disturbance of vision from physical or chemical agents, has led to a procedure to assess the potential of chemicals to damage ocular tissue. Draize and coworkers introduced in 1944 such a procedure, which was based on a standardized evaluation of the effects on the cornea, conjunctivae and iris by a scoring system. Eye irritation is specified by the production of reversible changes in the eye.

Both the OECD and the EC have published guidelines for eye irritation testing (OECD, 1981, guideline 405, update 1987; EC, 1984 and 1989a, guideline Annex V.B5). The procedures described in these guidelines are essentially the same as the Draize test from 1944.

The test material is instilled into the lower conjunctival sac of one eye of the test animal. The untreated eye serves as a control. The eyes are monitored for effects for a period long enough

to determine the reversibility or irreversibility of the effects. Normally, this period will not exceed 21 days.

In accordance with the skin irritation test, healthy adult albino rabbits are recommended in both the OECD and EC eye irritancy test guidelines. This recommendation is not only because of easy purchase and maintenance of rabbits under laboratory conditions, but also because rabbit eyes are relatively large compared with the body size and do not contain pigment, whereas the cornea is large in comparison with the total eye surface (Swanston, 1985). Furthermore, lachrymal flow in rabbits is low in comparison to man. The lachrymal fluid is more viscous and the blink reflex is nominal, which results in a slower clearance of the test compound from the eye. Eyes from several other species, e.g. primate, dog, rat, guinea pig, cat and chicken, have been used in studies into alternative animal models for eye irritancy testing (Beckley 1965, Beckley et al., 1969, Green et al., 1978, Freeberg et al. 1984, Seifried, 1986) for various reasons, such as costs, availability of the species, susceptibility and large differences in morphology and physiology. None of these species can be considered as an alternative to the albino rabbit. The current OECD and EC guidelines recommend the use of at least 3 animals. Extra animals may be required when the results of the original test are ambiguous. However, if severe effects are expected an eye irritation test on just one animal may be considered. When the results indicate severe irritation no further testing is needed.

The test substance is applied to the eye in doses of either 0.1 ml of undiluted liquids and pastes or 0.1 g of solids. There is some debate whether the applied volume results in exaggerated responses and thus in overestimation of the human hazard. Major arguments in this debate are the low capacity of the human eye - the volume of fluid normally residing in the human eye is 10 to 30 µl - (Swanston, 1985; Jacobs et al., 1987), and the possible triggering of a self-perpetuating and atypical process by large volumes, independent of the original chemical insult (Swanston, 1985). However, the eye irritancy test should be regarded as a potency test and in view of this amplification of the responses may be beneficial for the sensitivity of the test. If there are indications that the test material may cause "unreasonable pain", the guidelines recommend the use of anaesthetics during the time of application. However, it has to be ensured that neither the anaesthetic nor its dose cause differences in the reaction pattern to the test substance. Therefore, local anaesthetics with vasoconstrictive properties should not be used. Although literature reports are ambiguous with respect to this subject, one may conclude that the use of local anaesthesia may enhance the irritant effects in one way or the other (Arthur et al., 1986; Bell et al. 1979; Heywood & James, 1978; Johnson, 1980). This increase in response may be due to a prolonged contact between the eye and the test material, caused by the inhibition of the blink reflex at the time of instillation. Though, pain

upon instillation may indicate irritant properties of the chemical, the use of local anaesthetics is advocated to alleviate any unnecessary pain or distress at the time of instillation.

Irrigation of the eye of the test animal may be indicated for substances shown to be irritating. In this, the OECD and EC guidelines show some deviations. Whether these deviations are important is questionable. Irrigation has little or no effect on the irritation response, unless it is performed within 10 seconds of application of the test substance (Davies et al., 1976; Seabaugh et al., 1976). Even then the irritation response is influenced by a variety of factors, e.g. the volume of the rinsing solution and the duration of the rinsing procedure. It may be concluded that irrigation complicates the test, without adding valuable information. Furthermore, irrigation multiplies the number of animals, because the effect of irrigation has to be assessed in relation to non-irrigation. The need to test the irrigation effects on the irritant potential of a chemical, as is indicated by the guidelines, is even more paradoxical regarding the fact that the results of these studies are not considered in any hazard classification.

In accordance with the primary skin irritation test, substances with extreme pH values ($pH \leq 2$ or $pH \geq 11.5$) need not to be tested, due to their "probable corrosive properties". Murphy et al. (1982) and York et al. (1982), however, demonstrated that diverse substances with extreme pH values do not cause severe irritation in the rabbit eye. Therefore, the recent OECD guideline also recommends to take into account the buffer capacity of the chemical. Another category of compounds which is excluded from eye irritation testing includes materials which have demonstrated obvious corrosion or severe skin irritation. It is presumed that these chemicals will produce similar effects on the eye.

In contrast to the EC guideline, the updated OECD test guideline form 1987 indicates that test substances demonstrating potential corrosion or severe irritation in well-validated alternative studies may also be excluded from primary eye irritation tests. Such a statement acknowledges the relevance of alternative methods, including in vitro tests, in reducing dispensable in vivo tests. However, it has to be mentioned that at present none of the developed alternative methods is "well-validated".

Irritation is demonstrated by macroscopic changes of cornea, iris and conjunctivae. A prerequisite for evaluation is that these changes occur within 72 hours after exposure and remain for at last 24 hours. The changes vary from corneal opacity to vasodilation and swelling of the conjunctivae (chemosis) and iris lesions. Irritation is defined purely descriptive and the effects do not characterize the underlying mechanism. The various grades of the effects are recorded by a standard numerical system adapted from Draize et al. (1944)

A task group of experts of the European Chemical Industry Ecology and Toxicology Centre has reviewed the test guidelines on eye irritation (ECETOC, 1988)

4.3.2.4 *Results eye irritation test*

There are two main procedures to evaluate the responses in the eye irritation test. One approach, recommended by the EC, is scoring the intensity of the response in the different regions of the anterior eye during the first three days after exposure. If the mean score (determined at both 24, 48 and 72 hours after instillation) for either corneal opacity, iris lesion, conjunctival redness or chemosis, exceeds defined levels, the test substance is considered to be irritant. Nowadays most eye irritancy tests are performed with three animals. In this case defined levels have to be exceeded in at least two animals. When six or more animals are used the mean values have to be calculated over all animals tested. This approach does not reckon with other physiological disturbances of the eye like the occurrence of discharges and pannus and the irreversibility of the effects. Furthermore, the gravity of the reaction is not indicated. It is our opinion that all physiological and morphological alterations of the eye, persisting for at least 24 hours, should be considered as unwanted. Therefore chemicals inducing these effects should be classified as irritant (R38). In case of very persisting reactions, not disappearing up to the end of the test period, classification as irritant with risk of serious damage to eyes (R41) is indicated.

The other approach leads to a more gradual evaluation of the irritancy response. This approach is also based on the scoring scheme according to Draize et al. (1944), but incorporates the area as well as the degree of opacity of the cornea, iris and conjunctivae. Furthermore, this procedure merges the intensity and duration of the response up to 7 days. This scheme according to Kay & Calandra (1962) ranks chemicals in six classes: not and slightly irritating, irritating, and strongly, severely and extremely irritating.

Both above described approaches do not evaluate the irreversibility of the effects. The OECD guideline considers test substances inducing irreversible tissue damage as corrosive. However, such substances can not be classified in an appropriate manner according to EC criteria by which substances can only be classified as irritant or non-irritant.

The research for alternatives to animal eye irritation testing is enormous. The same arguments underlying the research for alternatives to animal skin testing are applied to the current efforts. A large number of in vitro models are developed; these models vary from cell cultures to enucleated eyes and non-biological systems. At present there are several validation studies in

progress. The main topic in these studies is the correlation between the in vitro data and the in vivo data. Studies (Goldberg 1983, 1985) show that a fine correlation can be obtained by several in vitro methods. For that reason in vitro methods are used in prescreening studies. Not only a high in vitro-in vivo correlation, but also the acceptance of a certain amount of risk is important for the introduction of in vitro methods in regulatory skin irritancy testing. In the OECD the debate about the incorporation of in vitro methods has just started. At the moment the most propitious alternatives are methods with enucleated eyes (Burton et al., 1981; Koëter & Prinsen, 1985) and tests with the chorioallantoic membrane from hen eggs (Lũpke & Kemper, 1986)

4.3.3 Sensitization

4.3.3.1 *Methods skin sensitization tests*

Knowledge about the skin sensitizing potential of substances is a public health concern. In the USA about 50% of occupational diseases are skin disorders, whereof 20% are of allergic origin (Keil & Shumnes, 1983; ECETOC 1990). The OECD guideline 406 (OECD, 1981) recommends seven skin sensitization tests to establish the skin sensitizing potential of chemicals. The updated EC guideline B6 (EC, 1989a) recommends only two sensitization tests. These predictive tests are based on studies of cellular immunology by Landsteiner and coworkers, which have been conducted largely in the guinea pig (Landsteiner & Jacobs, 1935, 1936: Landsteiner & Chase 1937, 1941, 1942).

Skin sensitization, also known as allergic contact dermatitis, is a T-lymphocyte mediated delayed hypersensitivity reaction. For a better understanding of the design of the sensitization tests a very brief description of the immunological events will be given. More detailed reviews of the mechanism are published by Abel & Wood (1986) and Thestrup-Pedersen et al. (1989). The immunological process can be separated into two aspects, the induction phase, in which the sensitization develops and the challenge phase, in which after renewed exposure to the same chemical, skin effects are evoked. In the induction phase a chemical penetrates the skin, in one way or the other, and interacts with Langerhans cells. These antigen-presenting cells migrate via the draining lymphatics to a lymph node. Through their dendritic shape the Langerhans cells are in close contact with T-lymphocytes in the paracontex of the lymph node. In combination with so called class II histocompatibility determinants the antigen specificity of a chemical will be imprinted in naive T-lymphocytes. A part of the induced T-lymphocytes, so called memory cells, are distributed all over the body. At this stage an individual is sensitized. The quiescent memory cells recognize the chemical as an antigen after a renewed exposure.

The T-cell will become activated and will release lymphokines and interleukines. These compounds mobilize other lymphocytes and macrophages. The released mediators activate the macrophages and consequently a local inflammatory reaction is initiated, which can be determined by erythema and/or oedema.

The sensitization tests can be subdivided into two groups. One group of tests using Freund's Complete Adjuvant (FCA) to enhance the sensitivity of the test methods and one group of non-adjuvant tests. FCA is a water-in-oil emulsion containing heat-killed Mycobacterium tuber-colosis. Adjuvant increases the antibody production and increases the number of lymphocytes and monocytes (Bühler, 1985). This results in an upgrading of the cellular response. There is a continuous debate on the toxicological validity of the use of adjuvant. Opponents of the adjuvant-techniques state that the evaluation should included a relevant route of exposure - FCA is injected intradermally - and that the experimental results should not be artifactual because of the test conditions (Bühler, 1985) In view of the aim of these sensitization tests, obtaining an indication of sensitization potential, these tests should be as sensitive as possible. In general FCA tests are more sensitive than non-adjuvant tests (Marzulli & Maguire, 1987).

The seven OECD-recommended sensitization tests are: the non-adjuvant Draize test (Draize et al. 1944, Draize 1959), Bühler test (Bühler, 1965; Bühler & Griffith, 1975; Robinson et al., 1989) and Open Epicutaneous test (Klecak et al., 1977), and the adjuvant tests: the Freund's Complete Adjuvant test (Klecak et al., 1977), the Guinea Pig Maximization test (Magnusson & Kligman, 1969, 1970), the Split Adjuvant Technique (Maguire & Chase, 1972; Maguire, 1973, 1975) and the Maurer Optimization Test (Maurer et al., 1978, 1979; Maurer, 1983). An additional, not widely used, "footpath technique" is also included in the Annex of the OECD-test guideline 406.

The OECD test guideline does not express any preference for the various sensitization tests. The recently updated EC-guideline B6 (EC, 1989a), however, recommends the Guinea Pig Maximization test (GPMT), bearing in mind that no single test method will adequately identify all substances with sensitizing potential to humans. The guideline suggests that, in certain cases, there may be good reason for choosing the Bühler test involving topical application rather than the intradermal injection used in the GPMT. In those cases, scientific justification should be given. Other tests may be used provided that they are well validated and scientific justification is given. It is stated that the Bühler test is sensitive enough to detect a moderate to strong sensitizer. The GPMT test is able to detect weak sensitizers (Klecak, 1987). Although the GPMT is the test of preference, it has to be remarked that FCA-treatment enlarges the animal distress.

Descriptions of the above mentioned methods are given by Klecak (1987). In view of the preference in the EC-test guideline, several pitfalls of the GPMT and Bühler test only will be discussed. An important prerequisite to induce chemical sensitization is penetration of the skin. The GPMT encounters this problem by both intradermal injection of the test substance (concentration of the test substance solutions is generally between 1 - 5 %) and epicutaneous application of the test substance in concentrations evoking some evidence of irritation. If the substance is not irritating, the application area is treated with 10% sodium lauryl sulfate in petrolatum to provoke a mild inflammatory response, which favours penetration and sensitization. Induction in the Bühler test has to be performed with concentrations of the test substance producing mild to moderate irritation in the majority of test animals. The irritant properties of a test substance has to be investigated in both methods in a primary irritation study. Both methods use occlusive topical application to enhance skin penetration. Some of the vehicles, like dimethyl sulfoxide (DMSO), facilitate skin penetration.

One frequently occurring problem with GPMT is the induction of severe local effects, i.e. tissue necrosis, at the intradermal injection site. Extended necrosis may hamper the antigen presentation mechanism. Marzulli & Maibach (1974) showed that the ability to induce sensitization is concentration dependent. A negative result of the skin sensitization test may be caused by an inadequate induction using a test substance concentration below threshold levels. This common mistake is often seen in Bühler tests, because the induction concentration is calculated with respect to the use concentration. (Klecak, 1987). Following challenge, sensitization is scored by means of subjective visual recording of erythema and/or oedema. The various skin sensitization testing methods use different grading scales and evaluation criteria. Furthermore, there is no commonly agreed prescription to enact a rechallenge. It is our view that in case of inconclusive results a rechallenge always should be performed using a naive control group. A rechallenge with lower dose may be considered when positive (irritation) response are detected in control animals.

When dyes are tested, difficulties may occur in reading the inflammatory response. Histopathological evaluation of the application site is recommended then. Since there is no clear-cut difference between the responses induced by sensitization or by irritation, histopathological examinations are more demanding than macroscopic evaluations. In irritant induced inflammation large numbers of neutrophils and a small number of macrophages can be detected. Often, damaged tissue can be observed. In a delayed hypersensitivity response an increased number of macrophages and basophils can be observed as well as the deposition of fibrin and spongiosis (Parish, 1986). In addition to the morphological changes, the differences in intensity of the inflammatory response will indicate the origin of the response. Therefore,

histopathological evaluation should be done in both tested and control animals.

Shillaker et al., (1989) have demonstrated that in most cases substances can be classified satisfactorily as sensitizer in GPM tests with reduced numbers of animals, i.e. 10 animals in the test group and 5 control animals. Although additional testing has to be performed if two or three e test animals show sensitization, this approach will ensure a considerable reduction in animal use. If properly conducted (see above remarks), such a "limited" test can be recommended.

4.3.3.2 *Results skin sensitization tests*

Guinea Pig Maximization Tests

Twenty four hours after removing the challenge patches the first reading of the reaction is carried out. The second reading is performed the next day. A positive reaction is indicated by red and swollen skin on the exposed sites. Skin reactions are evaluated with a four-point (0,1,2,3) scale. Assuming that a chemical does not provoke responses in the control group other than possibly slight redness in some animals, a chemical is regarded as a contact allergen when responses are scored ≥ 1. The frequency of sensitized animal in the test group is an indication of the sensitization potential. Although Kligman (1966) has developed a graded classification scheme, ranging from weak (grade I) to extreme (grade V), the EC considers a substance a sensitizer when it provokes in at least 30% of the tested animals a positive response (EC, 1991d).

It is our view that all skin reactions occurring at a higher incidence on test animals than in controls have to be considered as positive responses. Furthermore, not only frequency but also intensity and duration relative to controls have to be taken into account in the evaluation.

Bühler Test

In accordance with the GPMT, 24 hours after the removal of the patches the test site is examined for erythema and oedema for the first time. A second reading is made 48 h after challenge. The reaction is graded according a five point (0, ±, 1, 2, 3) scale. Reactions scored ≥ 1 are considered positive. The EC considers substances tested in a non adjuvant test to be sensitizers when a positive reaction is determined in at least 15% of the tested animals (EC, 1991d). Even an experienced investigator has some difficulty reading and evaluating the challenge test reactions in this procedure. Both control and test animals often show inflammatory responses. Differences in incidence, strength, duration and character of the responses relative to controls should indicate whether sensitization is involved. Absence of the positive

response does not indicate axiomatic absence of sensitization potential, because the possibility exists that the test concentration was below the minimal dose engendering sensitization (Klecak, 1987)

Allergic contact dermatitis is a complex multi-component process involving several organ systems. For that reason, it will be hardly possible to develop a practical in vitro model. However, several aspects of the sensitization process can be studied in vitro. Various studies on skin penetration, immunogen-Langerhans cell interaction, and lymphokines production have been performed. (Milner, 1987; Bronaugh & Maibach, 1987). However, the correlation between the effects on these separate components of the sensitization response and the ability of a substance to act as a sensitizer is ambiguous. Another alternative approach to the current sensitization tests is the use of mouse-models. Two models have shown to be promising. However, the Mouse Ear Swelling Test and the Local Lymph Node Assay should be further tested in an interlaboratory validation program to determine their sensitivity and specificity (ECETOC, 1990).

Human volunteer tests are often proposed as alternative methods to guinea pig sensitization test. Although these tests by-pass the basic problem of species extrapolation, the tests in our opinion enclose two major problems. The first problem concerns ethical considerations. Is it allowed to induce sensitization in human volunteers, regardless the volunteer's approval, which should be based on detailed information. It has to be borne in mind that the sensitization will be permanent. The other major problem is the necessity of large number of subjects in the test to obtain sufficient sensitivity. If human volunteer tests are used for classification and labelling, this need will irrevocable cause logistic and financial problems, regardless the problems to form reproducible representative test groups.

4.3.4 Subchronic toxicity

4.3.4.1 *Methods*

At base set level a 28-day test, using an appropriate route, should be submitted. Some notifiers submit a 90-day toxicity test instead, usually because of regulatory requirements outside the European Community. Both the OECD and the EC have produced test guidelines for 28-day and 90-day tests for oral, dermal, and inhalation exposure. With regard to the most appropriate route of exposure: see section 3.1. OECD and ECETOC (European Chemical Industry Ecology & Toxicology Centre) recently have issued publications summarizing and reviewing the internationally available subchronic toxicity test guidelines (OECD, 1991a; ECETOC, 1985)

together with a proposal for the updating of OECD guideline 407 concerning the 28-day oral test (OECD, 1991b). The EC Annex V guidelines have recently been updated (EC, 1989a).

Advice on the selection of doses can be obtained from the guidelines. It is important to emphasize that in general the highest dose (oral, dermal tests) or concentration (inhalation test) should produce toxic effects but no, or a few fatalities. At the lowest dose or concentration there should be no signs of toxicity. It is also important that the doses or concentrations administered are exactly known. Therefore, the stability of the substance in the medium of intake (diet, vehicle or air) must be known. In inhalation studies, the actual concentration should be measured in the breathing zone and should not vary by more than 15% in case of vapours. For solid particles and aerosols, measurement of the particle size should be made as often as necessary to assure consistency. A third point to emphasize is that a test report should always contain sufficient information on the identity and specifications of the test material.

4.3.4.2 *Results*

Concepts

Human data on new substances are not available and therefore the potential of a substance to cause effects following prolonged exposure is derived from results of tests on animals. One of the basic assumptions is that humans and mammals are similar in relative susceptibility to toxic chemicals. With respect to subchronic toxicity, it is the aim of the hazard evaluation to identify the most critical effect following prolonged exposure and to establish the relation between the dose, time, severity of effects (dose- and time-effect relation), and response (dose- and time-response relation) of the experimental animal. "The most critical effect" is the first effect, considered to be adverse, appearing when the dose of a substance is increased. "Dose" specifies the amount of a chemical administered. The term "effect" applies to the extent of biological changes and "response" can be defined as the incidence rate of effects. If possible, the data obtained need be evaluated vis a vis their significance to man.

For non-genotoxic effects, a certain substantial deviation from a statistically distributed normal value must be attained before a particular effect in an organism becomes manifest, resulting in a threshold dose for this effect. In some cases compensatory mechanisms, e.g. saturable detoxification by microsomal enzymes or feedback systems in endocrinology, may be responsible for the existence of a threshold dose. The subthreshold dose for the most critical effect in one test is called the Dose Without Effect (DWE) and is the highest exposure level without adverse, i.e. toxicologically relevant, effects. Effects regarded as non-adverse can still

occur below this Dose Without Effect. The threshold dose, i.e. the lowest exposure level in one test at which the most critical effect is occurring, is called the Lowest Effect Dose (LED). If more than one test is available, the overall evaluation leads to the selection of the most critical test. Unless a particular animal model is clearly not relevant to man, the most critical test is the most sensitive test on the most sensitive species, assuming that man is at least as responsive as this animal species. This approach applies if the tests are of comparable duration and quality. If not, the evaluation usually concentrates on the longer test and/or the test of better quality. However, in case of compensatory mechanisms effects observed in tests of short duration may not be seen in long-term tests: an example of such an effect is a decrease in thyroid hormones.

The DWE for the most critical test is widely known as the No-Observed-Adverse-Effect Level (NOAEL). The exposure level without any effect is called the No-Observed-Effect Level (NOEL) or No-Effect Level (NEL) and the lowest critical effect exposure level the Lowest-Observed-Adverse-Effect Level (LOAEL).

Derivation of a DWE

Guidance in selecting a DWE from a particular subchronic or chronic animal test can be obtained from publications of the Dutch Health Council (HC, 1985a and 1989), the International Programme on Chemical Safety (IPCS, 1978; 1987; 1990), and the US-Environmental Protection Agency (US-EPA, 1986). The guidance given below is partly derived from these publications and partly derived from experience gained in the hazard assessment of substances at the RIVM. In view of the limited base set, carcinogenic and genotoxic effects as well as immunotoxic and developmental effects usually cannot be taken into account at tonnage level 0 with respect to the determination of the DWE. The DWE will be based on pathological effects, i.e. non-genotoxic effects characterized by functional disturbances and/or morphological changes, and physiological effects, i.e. usually reversible effects such as reflexes and microsomal enzyme induction.

Crucial in the determination of a DWE is the differentiation between "non-adverse" and "adverse" effects and the decision whether any adverse effect observed is related to treatment, i.e. substance related. In subchronic toxicity testing the average values of selected parameters are compared to the average values of these parameters in untreated concurrent control animals. Adverse effects then could be defined in purely statistical terms as statistically significant ($P < 0.05$) changes relative to control values. This approach is too narrow: other factors need also be considered such as the presence or absence of a dose- and time-effect relation and/or a dose- and time-response relation, the biological relevance of an effect, the

reversibility of an effect, and the normal biological variation of effects such as can be shown, if certain conditions are met, by historical control values.

In view of the number of factors to be taken into account, it is not possible to present a complete decision tree for the derivation of a DWE. Expert judgement is an essential part of the evaluation process. Nevertheless, certain decision supporting rules can be described:

- Effects can be ranked in order of severity. Such an attempt was made by the US-EPA in 1986 (US-EPA, 1986) and the result, adapted to the hazard evaluation of subchronic tests using OECD or EC protocols and slightly expanded, is shown in table 2. The borderline between adverse and non-adverse effects can be drawn somewhere in the upper part of the table. It need be emphasized here that the degree of severity of an effect very much depends on duration and frequency of exposure and the site and characteristics of the particular change observed. Therefore, table 2 should be consulted cautiously.
- More weight is attached to changes in parameters which increase in severity and/or response with an increasing dose.
- More weight is attached to changes in parameters which are correlated to other changes observed. Examples are an increase in blood urea accompanied by an increase in kidney weight, an increase in liver weight accompanied by slight pathological changes such as fatty changes, or an increase in creatine phosphokinase combined with increases in lactate dehydrogenase and/or α-hydroxybutyrate dehydrogenase (indicative of myocardial damage). The lowest-effect-doses for these effects need not be coinciding. A survey of associations of changes in biochemical parameters with actions at particular target organs is presented by Gad & Weil (1982), Woodman (1988), and Stonard (1990).
- More weight is attached to changes in functional status of physiological or neurological processes, e.g. abnormal behaviour, if correlated to histopathological (peripheral nerve lesions) or biochemical changes (changes in blood acetylcholinesterase activity).
- More weight is attached to changes in, or related to, organs and tissues known to be a target of the substance. For example, a change in urinary volume certainly gains more biological significance if the kidney is known to be the target organ.
- More weight is attached to a parameter which shows a statistically significant change compared to control values than to a parameter which only shows a tendency towards a change. However, a tendency cannot be ignored in case a dose-effect or dose-response relation is apparent or in case other changes are found which could be associated.
- More weight is attached to effects which appear to be irreversible during or following exposure.
- Changes that occur in low incidence and perhaps are not even dose-related, but occur only in treated animals cannot be dismissed immediately as being not biologically relevant.

Table 2: Ranking of physiological and pathological effects in order of severity

Effect	Severity
Biochemical/haematological change with no pathological change and no change in organ weight; or a change in organ weight with no pathological and biochemical/haematological change	least severe ↑
Biochemical/haematological change with no pathological change and with a change in organ weight	|
Enzyme induction and subcellular proliferation or other changes on organelles but no other apparent effects	|
Biochemical/haematological change with slight pathological changes	|
Hyperplasia, hypertrophy or atrophy with a change in organ weights	|
Reversible cellular changes: cloudy swelling, hydropic change or fatty changes	|
Necrosis, or metaplasia with no apparent decrement of organ function; any neuropathy without apparent behavioral, sensory, or physiologic changes	|
Necrosis, atrophy, hypertrophy, or metaplasia with a detectable decrement of organ functions; any neuropathy with a measurable change in behavioral, sensory, or physiological activity; decreased body weight gain; clinical symptoms	|
Necrosis, atrophy, hypertrophy, or metaplasia with definitive organ dysfunction; any neuropathy with gross changes in behaviour, sensory, or motor performance	|
Pronounced pathological changes with severe organ dysfunction; any neuropathy with loss of behavioral or motor control or loss of sensory ability	| ↓
Death or pronounced life-shortening	most severe

Expert opinion is indispensable here.

- A change in a single haematological or biochemical parameter unsupported by other correlated haematological, biochemical or pathological changes may be biologically important e.g. in the case of acetylcholinesterase measurements. More weight is attached to such a change if it is statistically significant and dose-related. It should be realized that the study protocols usually only prescribe blood sampling at the end of the test. Therefore, time trends, which may help in the interpretation of certain effects cannot be observed.
- A statistically significant decrease in body-weight gain generally cannot be considered an adverse effect if coupled with a decreased food consumption.
- Organ weight changes should always be examined on an absolute and organ-to-body weight basis. Organ-to-body weight ratios (relative organ weights) can be misleading if a change in body weight occurs. Increased relative organ weights may be the result of adaptation to chemical stress: an increased liver weight may for example arise from a stimulation of protein synthesis which enables the liver to metabolize the foreign substance faster.
- The incidence of spontaneous changes is often highly variable among control groups of the same species and strain in different studies conducted. For reference data on biochemical and haematological values see for example Clampitt (1978) and Wolford et al. (1986). "Historical control values", i.e. data on the normal variation of a change in the test species, can be used in the interpretation of the biological significance of the changes observed, but should be used with great care. The historical control data ideally should be from the same species, strain, age, sex, supplier, and laboratory, and should come from contemporary control animals. If the authors of a report rely on historical control data in their interpretation of effects these should be provided together with the information necessary to assess their quality.
- In dermal tests two DWE's can be identified: one for systemic toxicity and one for dermal toxicity.

Further to the discussion on the differentiation between adverse and non-adverse effects the question can be raised, as is done at classification (section 4.5), at which dose or concentration "serious damage to health" is caused by the substance. According to the guidance provided by the EC (EC, 1991d; see Annex II) serious damage to health is considered to include death, clear functional disturbance or morphological changes which are toxicologically significant. Irreversibility of lesions is a key factor in this assessment. The response of cells and tissues to chemical injury at the intracellular level, i.e. biochemical, functional and structural changes, and/or extracellular level, i.e. metabolic and regulatory changes, can be categorized as either degeneration, inflammation or proliferation. The outcome of these pathological changes depends on the combinations in which they are occurring, their potency,

and their duration. Depending on these factors, initial injury such as mild cell degeneration or proliferation can, for example, regenerate to normal or eventually result in irreversible injury such as neoplasia. Therefore, even assuming that it is always possible to detect chemical injury at the intracellular level in a 28-day test - which is certainly not always a valid assumption - and taking into account the guidance given above and in Annex II, direct advice of experienced pathologists and toxicologists is essential for the correct evaluation of the degree of damage to health.

Uncertainties

As already illustrated above there are many sources of uncertainty in subchronic toxicity testing of substances. An effect may for example not be seen because the number of animals is too low, the time of observation too short, the dose level too low or too high - in the latter case the metabolic pathway may differ -, or simply because of inaccuracy or because the experimental design was too limited in scope. On the other hand false positives may be the result of low standard deviations. An excellent review on possible sources of uncertainty is presented in IPCS (1987).

Conversion factors

The approximate relation of ppm in the diet - or mg/kg diet - to mg/kg body weight is shown in table 3. If the conversion is applied to a 28-day test with rats, the conversion factor for young rats is chosen unless older rats are reportedly used. In case of a test duration of 90 days or longer the conversion factor for "old" rats is used.

Table 3: Conversion of mg/kg diet to mg/kg body weight
(Lehman, 1954)

Animal	Weight (kg)	Food consumed g/day	Conversion factor: ppm to mg/kg bw/day
mouse	0.02	3	0.15
rat (young)	0.10	10	0.10
rat (old)	0.40	20	0.05
guinea pig	0.75	30	0.04
rabbit	2.0	60	0.03

4.3.5 Genotoxicity

4.3.5.1 *Definitions*

In the present context, the broad terms genotoxic and genotoxicity refer to the induction by agents of potentially transmissible changes in the amount or structure of the genetic material of cells and organisms. These terms include the terms mutagenicity, clastogenicity and other indications of induced damage such as UDS (Unscheduled DNA Synthesis), SCE's (Sister Chromatid Exchanges) or mitotic recombination.

4.3.5.2 *Aim of testing chemicals for genotoxicity*

The genotoxicity tests are intended to screen chemicals for their potential genotoxic properties and their genotoxic action in germ cells and somatic cells, the latter being related to the carcinogenic properties.

Deleterious changes may occur spontaneously or be induced as a result of exposure to radiation or chemicals. In principle, exposure to genotoxic agents may be expected to result in higher frequency of mutations.

Classification and labelling of chemicals in relation to their mutagenic properties is important in ensuring that their safe manufacture, storage , transport, use and disposal can be accomplished. Criteria for considering that a substance should be regarded as potentially genotoxic to humans, and should therefore be classified, are given in the amended Annex VI to the Directive (EC, 1991d).

4.3.5.3 *Base set methods*

This section and section 4.5.3.4 will discuss the relevant test methods and will provide guidance on the evaluation of the results. For further guidance: see section 7.2.5.

Reverse mutation assay using bacteria

Methods: B13 and B14 (EC, 1989a), 471 and 472 (OECD, 1981)

- Bacterial reversion assays measures mutation in a series of amino-acid requiring auxotrophs of Salmonella typhimurium or Escherichia coli. In the Salmonella system (Ames test) the reversion is from histidine$^-$ to histidine$^+$, whilst in E. coli the reversion is from tryptophan$^-$ to tryptophan$^+$.
- Several methods for performing the bacterial reverse mutation assay have been described. The most widely used are the direct plate incorporation method and the preincubation

method. All plating for both methods should be done in triplicate and at least two independent experiments have to be conducted.

- Bacteria should be exposed to the test substance both in the presence and absence of an appropriate metabolic activation system. The most commonly used system is a cofactor supplemented post-mitochondrial fraction prepared from the livers of rats treated with enzyme-inducing agents. In some cases (oily substances and some azo dyes) it might be appropriate to utilize S9 from hamster (Prival & Mitchell, 1982).

In vitro mammalian cytogenetic test

Methods: B10 (EC, 1989a and EC, 1991e), 473 (OECD, 1981). Recently a guidance note has been issued regarding EC method B10 (EC, 1991e)

- The in vitro cytogenetic test is a test system for the detection of chromosomal aberrations in mammalian cells. The assays are designed to detect structural and not numerical aberrations.

  Although almost any cell line with a defined karyotype can be used for in vitro cytogenetic analysis, Chinese hamster cells and human lymphocytes are preferred for different reasons by many laboratories.
- The first test should differentiate between clear positives and negatives or equivocals. Only one harvest time may be used, in both the activation and nonactivation series of this first test, which has to be equivalent to 1-1.5x the normal cell cycle time after beginning of exposure.
- If the first test gives clear positive results and the substance has a structural alert (element in a chemical structure known to be associated with genotoxicity or carcinogenicity), further testing in vivo is indicated. A positive substance without structural alert has to be retested using the same protocol.
- When the first assay is negative or equivocal a second assay using two harvest times has to be initiated. The first harvest time should be equivalent to 1-1.5x the normal cell cycle after beginning of treatment and the late harvest should be at least one cell cycle later. From the late harvest only the highest dose and the untreated or solvent control need to be scored (unless positive results are obtained).

Further Comments:

- It has to be stressed that the treatment in the series without metabolic activation should be continuous!
- The highest dose should reduce the mitotic index by at least 50% (if possible).
- For negative controls it is sufficient to use only solvent controls (in duplicate).
- Tests that are performed using high concentrations of test substance, which alter the osmotic conditions or pH to an extent that chromosome integrity is disrupted, may be not

interpretable.

In vitro mammalian cell gene mutation assay

Methods: from EC (1986a) and no. 476 (OECD, 1981).

- Chinese hamster (CHO and V79) and mouse lymphoma (L5178Y) cells are the most commonly used cell lines for gene mutation studies. Most of these tests utilize one of three loci: HPRT (hypoxanthine guanine phosphoribosyl transferase), TK (thymidine kinase) or Na^+/K^+ ATPase (ouabain resistance). The HPRT and TK systems detect base-pair mutations, frameshift mutations and small deletions (see below) whereas the ouabain locus detects base pair substitutions and is probably suboptimal for frameshift mutations (frameshift mutations may result in complete loss of enzymatic function which in the case of ouabain will be rather lethal than mutagenic).
- The mouse lymphoma assay is considered to be the most sensitive. In addition, it seems that both point mutations and chromosome aberrations can be detected in this test system. Large colonies seem to be correlated with point mutations while small colonies seem to be derived from cells with major damage like chromosome aberrations.

Further comments:

- Cells should be exposed to the test substance both in the presence and absence of an appropriate metabolic activation system.
- For freely soluble substances pH and osmolality should be monitored during the exposure period.
- Results should be confirmed in an independent experiment.

4.3.5.4 *Post base set methods*

Micronucleus test/In vivo mammalian bone marrow cytogenetic test (chromosomal analysis)

Methods: B11 and B12 (EC, 1989a), 474 and 475 (OECD, 1981).

- These studies are used to evaluate the clastogenic activity of test substances in rodents. The micronucleus test detects damage to the chromosomes or damage of the mitotic apparatus while in the in vivo mammalian bone marrow cytogenetic test, the induction of structural chromosome aberrations can be investigated.
- The tests may be performed in two ways[1]:
  - Animals are treated only once and samples of bone marrow are taken at least twice, starting not earlier than 12 hours after treatment.

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[1] An alternative method for the micronucleus test, which has not yet been implemented in the guidelines, employs two administrations with an interval of 24 hours and only one sample time of 6 hours after the last treatment

- A repeated dosing schedule, indicated by pharmacokinetic and metabolic information on the substance, can be employed. Samples should be taken approximately 24 hours after the last administration of the chemical.
- An appropriate route of application should be chosen to ensure that significant absorption of the substance or its metabolites into the bloodstream occurs. Changes in the P/N ratio (in the micronucleus test) or mitotic index (in the bone marrow cytogenetic assay) are indications that the substance reaches the target tissue.

Further Comments:

- Many micronucleus tests may be considered inadequate by current standards in a considerable number of notifications submitted up to 1991. In particular, there is frequently no evidence of the chemical reaching the bone marrow in sufficient amounts. A guidance for further testing in these cases is given in a Strategy document issued by a special working group of the EC (see also section 7.2.6).

DNA damage and repair/Unscheduled DNA Synthesis (UDS) in mammalian liver cells in vivo

Method: new OECD guideline

- Induced DNA repair measured as unscheduled DNA synthesis (UDS) is currently accepted as an indicator of genotoxicity. The in vivo/in vitro method that is accepted now (OECD guideline), has been in use only for a short period of time. Therefore, the data base is relatively small.

  The UDS test measures DNA repair synthesis after excision and removal of a stretch of DNA containing a region of damaged induced by chemical agents. The test is based on the incorporation of ^{3}H-TdR (tritium labelled thymidine) into the DNA of the primary hepatocyte which can be detected by autoradiography. With this technique cells in S-phase are easily recognized by the dense labelling over the nuclei and only cells that show UDS are scored.

Further Comments:

- The endpoint "DNA repair" precludes the detection of carcinogens which act by mechanisms other than formation of repairable DNA adducts.
- In addition, the assay suffers from the limitation that test substances must be distributed to the target cells in sufficient quantities to elicit a detectable amount of DNA damage, particularly since weak responses tend to be obscured by cytoplasmic (background) grains.
- Evidence that a test substance or its metabolites reach the target cells (pharmacokinetic data) greatly strengthens the confidence in a negative UDS.
- It is possible that chemical substances inhibit DNA repair mechanisms, either directly or indirectly, this could also preclude detection of genotoxicity in the UDS assay.
- There are obviously a number of factors that confound the interpretation of negative data.

- Also the lack of sensitivity to detect some carcinogens remains an issue of concern with the in vivo UDS assay.

Tissue distribution of test chemicals in rodents

Method: proposed new OECD guideline

- Tissue distribution studies in rodents use generally inbred strains of rodents which are treated with a radiolabelled test substance. The uptake of labelled compound by a variety of organs is measured by scintillation counting and may be confirmed by whole body radiography.
- The organs chosen for the study may vary but should include at least: liver, kidneys, lung, stomach, small intestine, large intestine, bladder, blood, bone marrow, spleen and sex organs.
- Faeces and urine should be collected over the time period of investigation and measured for radioactivity.

4.3.6 Short-term aquatic toxicity

4.3.6.1 *Methods*

Two short-term aquatic toxicity tests are required, one with a fish species and the other with Daphnia, using the methods prescribed in the OECD guidelines for testing chemicals (OECD, 1981) or according to Annex V of the EC directive (EC, 1984). The Annex V methods have been updated recently. At the 10 tonnes/year level additional ecotoxicity tests may be requested (chapter 7). In that case the growth inhibition test with algae, the reproduction test with Daphnia, and the prolonged toxicity test with fish become relevant (OECD, 1981). This report only pertains to the base set tests. Other tests will be discussed in a follow-up report on the evaluation of new substances post base set.

Common sources of error in aquatic toxicity testing were reviewed by Lloyd (1986). These sources pertain to the concentration of the test substance, the recorded response, and the exposure period. It is important to know the actual concentration of the test substance. Homogeneous distribution is required. Losses may occur by precipitation, immiscibility, volatility, adsorption to vessel walls, biodegradation, and chemical degradation (hydrolysis, photolysis). The actual concentration, therefore, should be measured during the test. If a solvent is used to prepare a stock solution, a solvent control should reveal any toxicity caused by the solvent. The solvent may change the bioavailability of the test substance. Other sources of variation may arise from fluctuations in the test conditions such as changes in pH, dissolved

oxygen, hardness, temperature, light cycle, and salinity. The condition of the test species is another important variable, which should be routinely checked by using a reference substance.

If the concentration of a substance cannot be maintained within acceptable limits a semi-static or flow-through procedure should be adopted. Volatile substances, however, should be tested in a closed system. If a substance strongly adsorbs to vessel walls, saturation of adsorption sites by pre-incubation of the test vessels or rinsing of the test vessels with acetone before use may minimize losses.

4.3.6.2 *Results*

Based on past experience in the assessment of aquatic toxicity tests on new substances the following issues need be discussed:

In case solvents have been used to prepare stock solutions of poorly soluble substances the results should be evaluated carefully with regard to possible toxicity of the vehicle itself, the possible effect of the vehicle on the bioavailability and toxicity of the test substance, and the more problematic extrapolation to the aquatic environment.

In case the actual concentration of a substance has not been measured and other data on the stability of the substance in water are not available, the results based on nominal levels cannot be relied on because these may underestimate the true toxicity of the substance. Such a test cannot be evaluated.

In case actual concentrations are far below nominal ones without an acceptable explanation, the results cannot be evaluated because it must be assumed that an error was made in the administration of the dose or that a systematic error was made during analysis or calculation.

In case poorly soluble substances are tested at nominal levels far above the solubility limit and no effects are found, it can only be concluded that the endpoint investigated (LC_{50}, EC_{50}, NOEC) will be above this solubility limit. This is to be expected considering the fact that the substance will not be bioavailable. However, in some cases effects will be observed at or near the solubility limit and even a concentration-related response may be observed. Usually it is assumed that physical effects - e.g. blocking of or damage to the filter feeding system (setae) of Daphnia preventing food uptake, blocking of or damage to the gills of fish preventing gas exchange - are responsible for the effects observed. In general, if a concentration-related response is observed, the data obtained are evaluated in the same way as systemic effects,

although it is realized that the significance of such data to field situations is very uncertain (see also section 7.2.6).

It is recommended to evaluate the mortality rate in acute tests at different points in time. If the mortality rate decreases in time towards a value which is independent of time, the so-called incipient $L(E)C_{50}$, bioaccumulation of the substance in the test organism is suggested and further testing indicated. Delayed mortality may also become apparent and may require prolonged testing periods.

4.4 Evaluation of environmental fate data

4.4.1 Hydrolysis

4.4.1.1 *Methods*

The hydrolytical stability of the notified substances is assessed and evaluated according to the guidelines given by Annex V of the Directive (EC, 1984). Problems often encountered in assessing rates of hydrolysis relate to an aqueous solubility that is below the detection limit of the compounds investigated. On the basis of mainly qualitative structure-activity relationships (SAR's) published in the literature (for a review: Drossman et al., 1988), it is decided whether or not further testing of the rate of hydrolysis is appropriate for the notified substance. Using the SAR's, functional groups may be selected that are considered to be susceptible for hydrolysis. At the moment an increasing number of both qualitative and quantitative structure-activity relationships are becoming available that may be used for the description of the hydrolytical stability of notified substances (Peijnenburg, 1989 and 1991).

An important aspect of hydrolytically instable compounds is that generally upon introduction of a hydroxyl-group into the parent molecule, chemicals are formed having a lower K_{ow}-value. This implies that the products formed will be more soluble in water whereas they will also have a reduced bioconcentration potential.

4.4.1.2 *Results*

In setting up the final risk assessment for compounds having a half-life for hydrolysis, that is within the same order of magnitude as the length of the toxicity tests performed, it is important to note that the results of these tests may at least partially reflect the toxicity of the products formed upon hydrolysis. Thereupon, it may well be anticipated that in the aquatic environment

these compounds will be present as their hydrolysed products. This implies that in the final risk assessment the physico-chemical properties of the hydrolysed compounds need to be used as much as possible. At the moment, however, insufficient information is available to unambiguously determine the composition of all hydrolysis-products formed. Evidently, further research directed towards the identification of hydrolysis-products and the prediction of rates of hydrolysis is needed.

4.4.2 Biodegradation

4.4.2.1 *Methods*

For most organic xenobiotics, biodegradation is the most important removal process in the environment. Test guidelines for carrying out tests on biodegradation are given by the OECD (1981) and in the annexes of the EC directive (EC, 1979). In general four types of tests, all performed in an aquatic environment, may be distinguished:

1. Tests on ready biodegradability.
2. Tests on inherent biodegradability.
3. Simulation tests, simulating distinct environmental situations (for instance activated sludge, degradation under anaerobic circumstances).
4. Inhibition tests.

At level 0, one test for ready biodegradability needs to be submitted. In tests for ready biodegradability test conditions allow for a limited opportunity for biodegradation to occur. Therefore, any substance which is found to degrade when using these stringent methods, can be predicted to degrade rapidly in the aerobic environment and can be described as readily biodegradable. In Table 4 the relative stringency of the available ready biodegradability test methods are given. In the test methods stringency is related to the test conditions for degradation of the substance tested; the more stringent a test method is, the less favourable the test conditions are to degradation of the test substance.

In the test for inherent biodegradability the conditions used allow for a lower ratio of test substance to micro-organisms and prolonged exposure. If evidence of biodegradation is obtained the compound may then be considered inherently biodegradable, yet it must not be assumed that rapid or complete biodegradation will occur under environmental conditions. Two test methods are commonly used for determining inherent biodegradability; the Zahn-Wellens

Table 4: Ready biodegradability test methods and their relative stringency (EC, 1986c)

Test method	Duration (days)	Determinant[1]	Stringency[2]
Closed bottle	28	O_2 uptake[3]	most stringent
Modified MITI	28	O_2 uptake/DOC[4]	↑
Modified OECD	28	DOC/specific analysis	|
Modified AFNOR	28	DOC/specific analysis	↓
Modified STURM	28	CO_2/DOC/specific analysis[4]	least stringent

[1] DOC = dissolved organic carbon

[2] stringency: the most stringent test is less favourable to degradation of the substance.

[3] suitable for toxic substances

[4] suitable for poorly soluble substances

test method and the Semi-Continuous Activated Sludge test method (SCAS). In both methods the determinants are DOC as well as specific analysis of the test substance, with SCAS being less stringent than the Zahn-Wellens test method.

In inhibition tests the toxic effect of the test substance on a mixed micro-flora, is measured. The most common inhibition test method is given by the OECD (OECD, 1981) and in Annex V of the EC Directive (EC, 1984). Actually, this method is suggested as a toxic control experiment in ready biodegradability test run. In this method the toxic effect of the test substance on the degradation of acetate (a readily biodegradable compound), is measured. Another commonly used test method is the activated sludge inhibition test (OECD, 1981), in which the inhibition of the biochemical oxygen demand is being measured. Some other inhibition tests were evaluated by Struijs et al. (1985).

At the moment further research is carried out within OECD-expert groups on the development of additional and/or modified biodegradation tests. These 'new' tests include: additional biodegradation tests for volatile compounds and for compounds having a limited aqueous solubility, additional simulation tests for specific environmental circumstances (for instance low temperatures) and additional simulation tests for specific environmental compartments (for instance soil, air, groundwater, marine systems, aerobic and anaerobic sediments).

4.4.2.2 *Results*

Interpretation and evaluation of the results reported is carried out according to the guidelines given in the Directive (EC, 1979). On the basis of the results obtained, the notified substance is classified for biodegradation. Substances are considered readily biodegradable if one of the following criteria holds true (EC, 1991d and EC, 1986c):

a. If in 28 day biodegradation studies the following levels of degradation are achieved:
   - In tests based upon dissolved organic carbon: 70 %.
   - In tests based upon oxygen depletion or carbon dioxide generation: 60 % of the theoretical maxima.

   These levels of biodegradation must be achieved within 10 days after the start of degradation, which point is taken as the time when 10 % of the substance has been degraded.
b. Or if, in those cases that only data on Chemical Oxygen Demand (COD) and Biological Oxygen Demand after 5 days of incubation (BOD_5) are available, the ratio BOD_5/COD is greater than or equal to 0.5.
c. Or if other convincing evidence, to be judged by an expert on an ad hoc-basis, is available to demonstrate that the substance can be degraded in the aquatic environment to a level >70 % within a 28-day period.

In the final risk assessment, in which all compounds are assumed to pass a municipal Waste Water Treatment Plant (WWTP) upon discharge to the aquatic environment (de Nijs et al., 1988), a pseudo first order biodegradation rate constant (k_{deg}) is derived for compounds classified as being readily biodegradable. As reported by Struijs et al. (1991) it is generally accepted that a compound that has passed one of the stringent OECD tests for "ready biodegradability" will also be mineralized (> 90 %) in a WWTP, even if biodegradation is the sole removal mechanism; this despite the fact that according to the OECD test hierarchy (OECD, 1981) the tests on "ready biodegradability" are not meant to simulate biodegradation in WWTP. Based on mass balance equations (Struijs et al., 1991), a value of k_{deg} of 3 h^{-1} is assigned to compounds shown to be readily biodegradable; for compounds not being inherently biodegradable, the value of k_{deg} is set equal to 0.

4.4.3 Bioaccumulation

4.4.3.1 *Methods*

The potential for bioaccumulation of notified substances is assessed according to the guidelines given by the OECD (1981). The key elements for the evaluation of the bioaccumu-

lative potential of chemicals are the K_{ow}-value, the aqueous solubility, the fat solubility, the degradability (including metabolism) and the molecular weight (size) of the chemical. In order to get a first indication of the bioaccumulative potential of organic substances the bioconcentration factor (BCF) of the test substance is calculated, using the following Quantitative Structure Activity Relationship (QSAR) developed by Veith & Kosian (1983):

$$\log BCF = 0.79 * \log K_{ow} - 0.4 \quad (1)$$

It is noted that in the near future this relation will be replaced by the QSAR of MacKay (1982) as recommended by Slooff et al. (1992):

$$BCF = 0.05\ K_{ow} \quad (2)$$

In general there may be quite large deviations between calculated and measured BCF's. Thus Kristensen and Tyle (1990) reported deviations between calculated and measured BCF's for fish by factors ranging from 6 - 90. Also, equation 1 is valid only for non-dissociating organic chemicals with a log K_{ow} between 2 and 6.

4.4.3.2 *Results*

In general a compound is considered to have a strong potential for bioaccumulation if all of the following criteria are fulfilled (Kristensen and Tyle (1990):

1. The log K_{ow}-value exceeds 4.3; this usually means that the aqueous solubility is limited, whereas the compound is soluble in fat. Given the uncertainties denoted above, a K_{ow}-value of 4.3 corresponds to a BCF of 1000 (equations 1 and 2).
2. The compound is not readily degradable in water and metabolism does not occur.
3. The compound does not ionize in water.
4. The compound is not a metal or an organo-metal.
5. The molecular mass is below approximately 700 dalton.

A compound is considered to have a moderate potential for bioaccumulation when the criteria 2 - 5 given above are fulfilled and when the logK_{ow} value ranges from approximately 3 - 4.3 (equation 1), corresponding to a BCF ranging from 100 to 1000. If the log K_{ow}-value is below 3, the compound is considered to have no bioaccumulating potential. Finally, for superhydrophobic substances (log Kow > 6) for which the QSARs developed are not valid, contributions to bioaccumulation through other compartments than water (especially food) will be significant. These compounds too are considered to have a strong potential for bioaccumulation (Connell,

1990). Given the reported regression equations of log K_{ow}-values versus log BCF-values and given the uncertainties upon application of these equations, a log K_{ow}-value of 3 has been agreed upon as a cut-off value for further testing of bioaccumulation in fish for organic chemicals which meet the criteria 2 - 5 given above (Esser & Moser, 1982).

Recently methods for the assessment of bioaccumulation have been critically evaluated by Kristensen and Tyle (1990); most methods are based on relationships between bioconcentration and the octanol-water partition coefficient. It was concluded that for further development of estimation methods of bioaccumulation, more reliable test data on log K_{ow}-bioaccumulation correlations are needed for various chemical classes and aquatic species or taxa. Besides, correlations based on other physico/chemical properties than K_{ow}, need to be developed. The main conclusion, however, was that the present OECD test guidelines need to be updated. The update should concentrate on other aquatic taxa than fish (e.g. mollusca, crustacea, annelida). More attention needs also to be paid to biomagnification.

In view of the present activities in the field of bioaccumulation it is to be expected that in the near future additional methods will become available for the estimation of the potential of bioaccumulation (biomagnification) of chemicals. These methods will be used to further improve the evaluation of the potential for bioaccumulation of notified substances.

4.5 Classification and labelling

The criteria for classification and labelling are laid down in Annex VI of the European Directive 79/831/EEC. Symbols and indications of danger for the label are presented in Annex II of the Directive. Both annexes recently have been amended (EC, 1991d). The object of classification is stated to be: "to identify all the toxicological, physico-chemical, and ecotoxicological properties of substances and toxicological and physico-chemical properties of preparations which may constitute a risk during normal handling or use. Having identified any hazardous properties the substance or preparation must then be labelled to indicate the hazard(s) in order to protect the user, the general public and the environment". It is stressed here that classification and labelling pertains to intrinsic properties revealed in the hazard identification process, but not to hazard or risk assessment. Exposure considerations are outside the scope of this exercise.

In this section several classification issues will be discussed which cannot be resolved completely using Annex VI.

Oxidizing properties

If a combustible substance melts, its mixture with cellulose may burn more rapidly than the reference mixture in the test for oxidizing properties because the melting substance spreads over the large surface of the cellulose. A false positive result is then obtained which incorrectly leads to classification of the substance as oxidizing (symbol "O", R8). If incorrect classification is suspected, e.g. on the basis of the chemical structure, the test should be repeated in a nitrogen atmosphere or by mixing the substance with an inert substance such as kieselguhr instead of cellulose.

Acute inhalation toxicity

Substances shall be classified as harmful and assigned the symbol "Xn" and the indication of danger "harmful by inhalation" (Risk phrase R20), if the 4-h LC_{50} exceeds 2 mg/l and is less than or equal to 20 mg/l. If tested by the inhalation route, a solid or liquid substance will often be tested up to the highest concentration of respirable particles or aerosol technically feasible. This concentration frequently does not exceed the classification limit of 20 mg/l. If no mortality is observed, classification is then not required. In case of mortality over 10%, classification is deemed necessary in view of the uncertainties involved in acute toxicity testing unless data show a shallow dose-response curve.

Skin and eye irritation

Classification of new chemicals as irritants has been discussed in sections 4.3.2.2 and 4.3.2.4.

Sensitization

Classification of new substances as sensitizing agents has been discussed in section 4.3.3.2.

Subchronic toxicity

Based on subchronic tests a substance can, among others, be assigned the risk phrase R48 (danger of serious damage to health by prolonged exposure, R48). This phrase shall be assigned in accordance to the following criteria: "serious damage (clear functional disturbance or morphological change which has toxicological significance) is likely to be caused by repeated or prolonged exposure by an appropriate route". The dose ranges in which these effects are observed determine classification as "toxic" (symbol "T") or "harmful" (symbol "Xn"). The assessment whether there is "serious damage to health" is an even more difficult issue than the determination of the "Dose Without Effect" (section 4.3.4). Guidance is given by the EC (EC, 1991d) in Annex VI (Annex II to this report) and by the discussion on the nature of effects in section 4.3.4. It is noted that irreversibility of changes is a key factor in assigning R48; this issue often cannot be fully resolved on the basis of a 28-day test.

Genotoxicity

Criteria for classification of a substance as potentially genotoxic to humans are provided in Annex VI to the Directive (EC, 1991d). However, see also chapter 7 on testing strategies (section 7.2.5).

4.6 Evaluation of safety data

A notification should contain information regarding recommended methods and precautions concerning handling, storage, transport, fire, and other dangers, emergency measures in the case of accidental spillage and in the case of injury to persons, information regarding packaging, and information on possibilities to render the substance harmless. This information is assessed for completeness and correctness. It should be in line with the other data in the notification, with the hazard identification and the resulting classification and labelling, and with national regulations. Examples of cases are:

- no information on first aid or fire extinguishing agents
- a recommendation to rinse eyes with water when this measure has been shown to be ineffective in the eye irritation test with rabbits
- a recommendation to induce vomiting in case of a corrosive substance
- a recommendation to use freons (halons) as a fire extinguishing agent which is not in accordance with the national policy to reduce the use of substances which degrade the ozone layer.

5. EXPOSURE ASSESSMENT

5.1 Indirect exposure

5.1.1 Introduction

Indirect exposure of new substances takes place through the following pathway:

Emission -> Distribution -> Concentrations -> Exposure

An assessment of the exposure of target organisms includes therefore:

- an estimation of the emission into the environment;
- an estimation of the distribution of these emissions in the environment, resulting in an estimation of concentrations in the environment (PEC = Predicted Environmental Concentration).

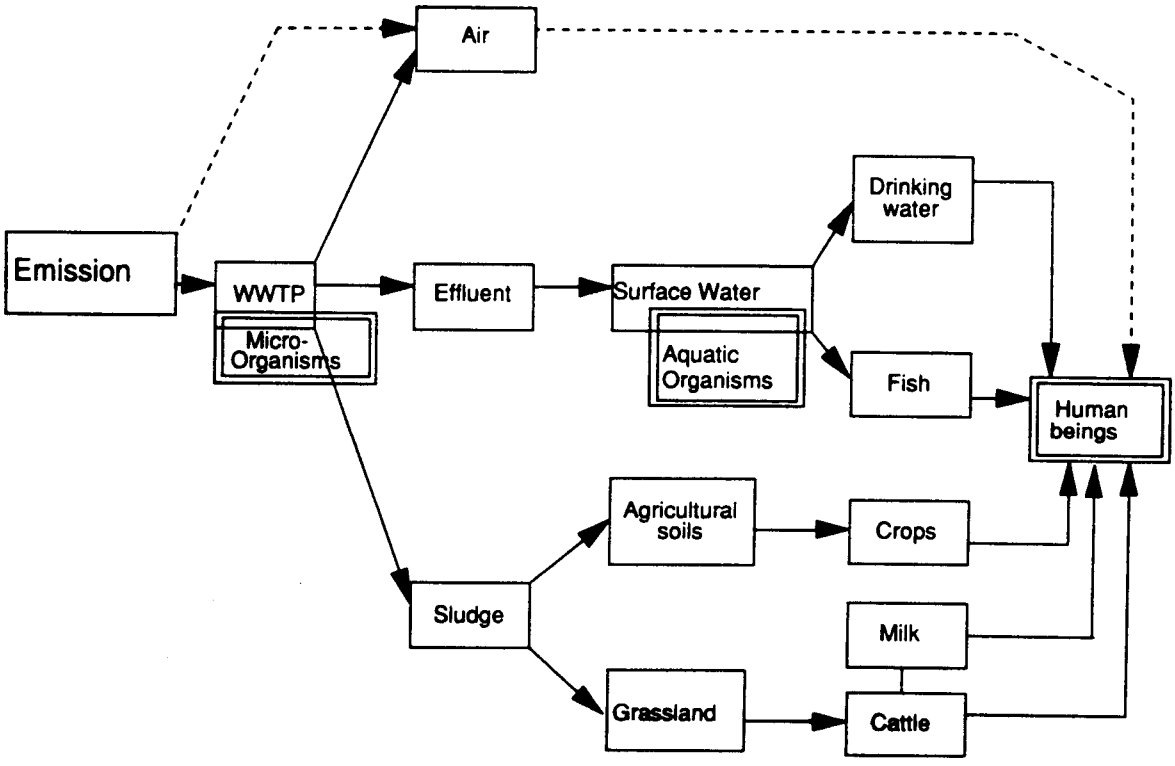

Fig.5: Routes of exposure in the Assessment System for New Substances

In order to assess risks of new chemical substances, an Assessment System for New Substances (Dutch acronym: BNS) has been developed by the RIVM. This system includes the

estimation of the distribution of the substances. Emissions are estimated with an expert system (PECker). An overview of these systems is given in Roghair (1988b), de Nijs et al. (1988), Toet et al. (1991) and van der Poel (1991). Figure 5 gives a schematic design of the routes which are included in the systems.

5.1.2 Emissions

The expert system PECker (van der Poel, 1991) estimates emissions of new substances according to the results of extensive industry category studies concerning losses during production, processing, use, waste processing and recycling of defined groups of substances. Seven groups of organic chemicals are distinguished in the estimation system (in brackets the reference to the underlying study):

- textile dyes (Ros, 1985);
- paper chemicals (Ros & Berns, 1988);
- photochemicals (Ros & Bogte, 1985);
- additives for metal working/hydraulic fluids (van der Poel & Ros, 1987);
- intermediate products (Ros et al., 1990, Ros & van der Poel, 1989);
- plastic additives (in preparation).
- general, for all other chemicals.

In the present state of the system, only losses to water are estimated. An estimation is made of the number of emission sources, and the number of days that emission will take place. Results of the program are the emission per source and per activity (production, compounding, processing, use and recovery), and the uncertainty factor of these emissions.

5.1.3 Distribution

The basic philosophy behind the estimation of environmental concentrations is summarized as follows:

- Given the relatively small production volume (1-100 tonnes/year), the local distribution near a point source should be considered;
- The estimation should not be location specific. This implicates a construction of a 'standard environment', which reflects average circumstances in The Netherlands;
- Though estimations are made for this standard environment, variability due to local deviations should be accounted for through the approximation of uncertainty in the concentrations calculated.

The main assumptions made in the Assessment System for New Substances with regard to distribution of substances in the environment are:

- Steady-state concentrations in the environment are regarded, although it may take many years before these concentrations will be reached;
- Target organisms are: micro-organisms in a waste water treatment plant, aquatic organisms in surface water, and human beings.

In order to estimate the exposure of the target organisms, the main routes of chemicals in the environment to these organisms are determined and modelled according to accepted concepts. These routes are shown in figure 5. Some points of this scheme which can be subject of discussion are summarized below:

- Emissions are considered to originate from point sources, which produce a continuous load toward water, air and soil. In the present state, only emissions to water are regarded. If emissions vary through time, the highest occurring emission is chosen;
- All water emissions are assumed to be collected and passed through a waste water treatment plant (WWTP). Depending on the characteristics of the chemical, removal may occur by biodegradation, adsorption to sludge or volatilization (see Struijs et al., 1991). The effluent is diluted with surface water (de Greef & de Nijs, 1990). Sludge from the WWTP is used as fertilizer on agricultural land and grassland;
- Only surface water is used in the preparation of drinking water. In the present state of the system, only the particulate fraction of chemicals is removed during preparation (Roghair, 1988b);
- Concentrations in human food are estimated by means of bioconcentration factors from surface water to fish, and from soil to agricultural crops, meat and dairy products. Bioconcentration factors are estimated on the basis of the octanol/water partition coefficient of a substance (Roghair, 1988b; de Nijs & Vermeire, 1990);
- The total daily intake by human beings is the sum of the intake of different food media: drinking water, fish, crops (vegetables, fruits, grains), meat and dairy products. The average intake of these products in The Netherlands is used. It is assumed that all food and drinking water originates from 'contaminated' areas in The Netherlands (de Nijs & Vermeire, 1990).

A validation study of the Assessment System for New Substances has been carried out, using measured ranges of environmental concentrations of 25 chemicals (Toet et al., 1991). The results show a reasonable agreement between measured and calculated concentrations in effluent, surface water and drinking water. Large deviations in concentrations in cattle and plants are found, though the total daily human intake agrees fairly well with measured values. The source of the large deviations is mainly to be found in the large over- and

underestimations of bioconcentration factors.

5.1.4 Uncertainty analysis

The uncertainty in values of constants, parameters and expert estimates used in the Risk Assessment System for New Substances is large. The estimation of emissions includes many uncertainties about production mode, use, waste disposal etc. In the estimation of environmental concentration, large uncertainties are included in the estimation of bioconcentration factors. Large uncertainties also originate from the variability of the actual environment, compared to the 'standard' environment used in the Assessment System for New Substances.

The Assessment System for New substances includes some kind of uncertainty assessment of the results. This has not yet been worked out completely. For the distribution module of the Assessment System for New Substances, only the exposure of aquatic organisms is subject to an uncertainty analysis. This analysis accounts for the probability distribution of model parameters, resulting in an uncertainty in model predictions (Slob & de Nijs, 1989).

5.1.5 Discussion and future developments

The assessment of indirect exposure is inevitably complicated. First, the information on new substances (physical, chemical) is scarce. Second, emission and distribution in the environment can only be roughly modelled. Third, the assessment is further complicated by the use of a 'standard environment', which should account for average conditions in The Netherlands.

In the near future, the emission estimation system PECker will be extended with estimations of losses to air and soil.

The distribution module of the Assessment System for New Substances in the present state, includes the main routes toward the target organisms. The purification of drinking water is still being studied. Additional pathways, which are under investigation, are:

- Emission to air -> human beings
- Emission to air -> deposition on soil -> plants/cattle
- Soil -> ground water -> drinking water

The uncertainty in estimated exposures is large. Goal of the Assessment System for New Substances is to quantify the uncertainties in the estimations. In the present state, this has only partly been implemented. In future, the uncertainty analysis will be extended to the indirect

exposure of human beings. To account for variability in all model parameters, a Monte Carlo technique will be implemented in the system. An important point of action is also the distinction between uncertainty in the model descriptions (for example the estimation of bioconcentration factors from octanol/water partition coefficients) and uncertainty caused by variability in the environment.

5.2 Direct exposure

The public at large usually will hardly be exposed to new substances due to handling as such. Normally, a new substance will be part of a formulation or an article. If the new substance is part of an article (e.g. an additive to a polymer, a component of printing ink on paper) the user will be exposed to the substance because of direct contact. This contact can be dermal as well as oral, for instance by drinking from a plastic cup containing the new substance as part of the polymer. Exposure is due to migration, evaporation, and/or leaching and is influenced by the fate of the substance and human behaviour. Unfortunately very little is known about the release of new substances from products. Only for a few formulations such as cosmetics the degree of direct exposure may be estimated quantitatively. In most cases qualitative exposure estimates only are possible, mainly concentrating on the probability of dermal exposure and irritating and sensitizing effects on the skin. Currently an attempt is made at the RIVM to develop methods for a systematic quantitative estimation of direct exposure of consumers to substances.

6. HAZARD AND RISK ASSESSMENT

6.1 Hazard/risk to man

6.1.1 Indirect exposure

6.1.1.1 *Introduction*

Human beings living in a potentially contaminated area may be exposed indirectly to chemical substances via food, drinking water, and directly via air. The estimation of environmental concentrations and the estimation of the total daily intake of man using the Assessment System for New Substances has been described in 5.1. Man here is assumed to consume contaminated food and drinking water only.

6.1.1.2 *Extrapolation to No-Effect Concentrations*

The only, admittedly limited, model available to estimate the effects from long-term intake of substances by man is the rat in a 28-day test. The oral route usually is preferred. In this test groups are dosed by gavage once daily. The substance is given as such or via the diet or in a vehicle (e.g. water with or without dispersing agents, corn oil). A Dose Without Effects is derived as explained in section 4.3.4.1.

6.1.1.3 *Hazard/risk assessment*

The highest dose without effect (DWE) from the most critical test (section 4.3.4.1) is, without further extrapolation, directly compared to the predicted total daily intake (Roghair, 1988b). The resulting "margin of safety" indicates the degree of risk. This approach, rather than a more rigid safety factor approach, allows greater freedom of expert judgement. Depending on the incidence, type and severity of the effect, the dose-effect and dose-response relations observed and the availability of other data on the toxicological profile of a substance, the resulting margin of safety can be judged to be sufficient or not. The margin of safety should also account for uncertainties resulting from intraspecies variations, i.e. variations in the sensitivity among individuals of one species, uncertainties resulting from interspecies variations, i.e. variations between the species man and the test animal, and uncertainties resulting from differences in the exposure scenario, e.g. short-term versus long-term exposure and continuous versus intermittent exposure. Last, but not least, the margin of safety should allow for the

uncertainty of the estimated daily intake.

Depending on the availability of data which in the opinion of experts may shed some light on the magnitude of the margin of safety, it seems appropriate to demand a margin of safety of 1000 to 10,000 between the calculated daily intake and the 28-day DWE-rat for non-genotoxic compounds.

6.1.2 Direct exposure

The likelihood for man of becoming exposed, the possible routes and the degree of exposure should be related to toxicity data for the relevant route(s) of exposure. For dermal contact the results of skin and eye irritation tests and skin sensitization tests are also relevant. However, it should be borne in mind that these tests reflect the intrinsic properties of the substance. The conditions of these tests are optimized in order to obtain a maximum response. As already reported in section 5.1.2 attempts are currently made at the RIVM to develop methods for a systematic assessment of the hazards from direct exposure of consumers.

6.2 Hazard/risk to the environment

6.2.1. Hazard/risk to target organisms

6.2.1.1 *Introduction*

Organisms living in a potentially contaminated area are at risk of being intoxicated, causing effects like inhibition or death. The hazard assessment is carried out by comparing environmental concentrations with no-effect concentrations for the target organisms selected: micro-organisms in a waste water treatment plant and aquatic organisms. The target organism man was discussed in the previous section.

Hazard assessment for new chemicals is carried out by the Assessment System for New Substances, which estimates environmental concentrations, no-effect concentrations and hazard quotients.

The estimation of environmental concentrations has been described in 5.1. No-effect concentrations are estimated by means of extrapolation of ecotoxicological test results. Hazard levels are calculated as a quotient of predicted environmental concentrations and no-effect concentrations. Risk levels are calculated by combining the hazard assessment results with an

uncertainty analysis.

6.2.1.2 *Extrapolation to No-Effect Concentrations*

The notification contains data on the short-term toxicity of the new substance for some water organisms (usually fish and waterflea, Daphnia). Incidentally, a test with algae is also provided as well as an inhibition test for bacteria in a waste water treatment plant. The results are LC_{50}, EC_{50}, and/or IC_{50} for single species (chapter 4). These results have to be extrapolated to chronic no-effect concentrations for aquatic ecosystems and micro-organisms.

The extrapolation of single species tests for aquatic organisms is carried out according to the modified EPA-method (OECD, 1990). A factor 10, 100 or 1000 is applied to the lowest L(E)C50 or NOEC, depending on the availability of data (table 5). Recently the method was reviewed by Slooff et al. (1992). It is recognized that the method has no scientific basis.

Table 5: Extrapolation of aquatic toxicity data to the aquatic ecosystem

Information available	Assessment factor[1]
Lowest acute $L(E)C_{50}$	1000
Lowest $L(E)C_{50}$ for at least representatives of algae, crustaceans and fish	100
Lowest NOEC for at least representatives of algae, crustaceans and fish	10

[1] If the required information is only partly present the lowest value obtained upon application of the various factors is chosen as the no-effect concentration for the aquatic ecosystem

IC_{50} values for micro-organisms are extrapolated to IC_0 values (no inhibition) by applying a factor 10, as estimated by Roghair (1988b).

6.2.1.3 *Hazard/risk assessment*

The environmental concentrations, as calculated by the Assessment System for New Sub-

stances, are expressed as median values. For aquatic systems, an uncertainty factor is also calculated. No-effect levels are estimated by rough extrapolation methods, without further uncertainty analysis.

A median hazard level is calculated by simply calculating the quotient of the environmental concentration and the no-effect concentration. For both aquatic organisms and micro-organisms, there is a degree of risk if this quotient exceeds unity.

Additional to the median hazard level, the probability that the hazard quotient exceeds unity can also be calculated for micro-organisms and aquatic organisms. Accounting for variability in environmental conditions and uncertainty in estimated variables, this is a better measure for the risk level. For man, an uncertainty analysis has not yet been implemented.

6.2.1.3 *Discussion and future developments*

The assessment of hazard and risk to target organisms is limited by the amount of toxicity data in the notification of new chemicals. This leads to high extrapolation factors, to ensure that accurate no-effect concentrations can be estimated (especially for aquatic organisms). For micro-organisms in a waste water treatment plant, only scarce data are available. Chronic effects on human beings can only be estimated from subchronic toxicity tests on rats.

In the future, more accurate extrapolation methods may become available. A further uncertainty analysis of the distribution of chemicals and the exposure of human beings will lead to a more probabilistic concept of "risk level", as is already the case for aquatic organisms.

6.2.2 Atmospheric hazards

After its release in the atmosphere, the primary emitted compound, or a secondary formed degradation product may have adverse effects on man or ecosystems. The potential risks by direct (inhalation) or indirect exposure for man have already been discussed in section 6.1. In this section emphasis is on the (potential) contribution to environmental problems related to the atmosphere. In more particular, the possible impact of new substances on

- climatic change and
- atmospheric ozone, both in troposphere and stratosphere and
- acidification

will be shortly discussed, a more detailed discussion is given in de Leeuw (1992).

The potential environmental risk of a substance is largely determined by its atmospheric residence time. The processes which govern the residence time are dry and wet deposition and chemical transformation. Estimation procedures of dry and wet deposition velocities are summarized by Noordijk & de Leeuw (1991). The following processes may contribute to the atmospheric chemical degradation :

- photolysis
- reaction with hydroxyl-radicals (OH-radicals)
- reaction with ozone (O_3)
- reaction with nitrate-radicals (NO_3-radicals) and other photochemically generated species.

With exception of photolysis, structure-activity-relations (QSARs) or related procedures are available from literature to estimate the reaction rate constants (for further discussion see de Leeuw, 1992). When the gas-phase UV-spectrum of a new substance is available an upper limit of its photolysis rate can be estimated by combining the measured absorbances with information on the solar spectrum which is available in tabulated form (see e.g. Frank & Klöpffer, 1988) or from radiation transfer models. The application of radiation transfer models (see e.g. Demerjian et al., 1980) has the advantage that the photolysis rate can be estimated at various altitudes, e.g. at ground level, in the free troposphere and in the stratosphere.

In global and continental atmospheric environmental problems like climatic change or the deposition of toxic or persistent compounds, a large number of compounds emitted from numerous sources on a continental or global scale is involved. With respect to the emissions of "old" substances, the contribution of "new" substances will be marginal. Obviously, this is also the case for any additional emissions of the "old" substances. Therefore, as a starting point in the assessment of the atmospheric impact of new substances it is more appropriate to use the concept of "hazard potential" rather than the absolute emission amounts. The "hazard potential" is the harmfulness of an emitted amount of a new compound expressed relative to the harmfulness of the same amount of a reference substance. The hazard potential for climatic change, atmospheric ozone and acidification may form an instrument to regulate the (world-wide) emissions of new substances.

Climatic change

The impact of a new substance on global warming depends on its infra-red (IR) absorption characteristics and its atmospheric lifetime. When the new substance shows absorption bands in the so-called atmospheric window (8.5-11 μm, a spectral region where the absorption by other gases is minimal) it must be marked as a potential greenhouse gas. In this case, it is necessary to estimate the "global warming potential" (GWP). The GWP may be defined as the ratio of calculated warming for each unit of mass of a gas emitted into the atmosphere relative

to the calculated warming for a mass unit of the reference gas CFC-11. The GWP provides a measure of the cumulative effect on the radiative balance over the chemical lifetime of each mass unit emitted in the atmosphere. It also provides a measure of the maximum calculated effect of a compound compared to the maximum calculated effect of an equal amount of CFC-11. The concept of GWP has proven its applicability in the assessment of the climatic impact of chlorofluorocarbons (CFCs) and their proposed substitutes hydrogenchlorofluorocarbons (HCFCs) (WMO, 1989).

For a first approximation of GWP-values estimates of atmospheric lifetime and the IR absorption strength in the interval 800-1200 cm^{-1} are needed (Brown et al., 1989). For species with lifetimes less than 1-2 year GWP-values less than 0.03 are in general expected.

Atmospheric ozone

As for atmospheric ozone, a discrimination between the possible impact on stratospheric ozone (i.e the ozone layer at an altitude of ca. 15-50 km) and the potential of ozone formation in the troposphere (the lower part of the atmosphere up to about 12 km) has to be made.

Stratospheric ozone

The new substance may have an effect on stratospheric ozone if

- it contains Cl or Br substituents, and
- the atmospheric lifetime is long enough to allow for transport to the stratosphere.

The potential risk of a new compound can be estimated from its ODP-value. The ozone depletion potential (ODP) is defined as the ratio of calculated ozone column change for each mass unit of a gas emitted into the atmosphere relative to the calculated depletion of the reference gas CFC-11. Column ozone is the total amount of ozone between the earth's surface and space or, mathematically expressed, it is the integral of the ozone concentration through the entire atmosphere. As stratospheric ozone contributes to about 90% of the total ozone column, the ODP is a useful measure of the potential for each compound to affect the stratospheric ozone layer. The ODP provides a measure of the cumulative effect on the ozone column over the chemical lifetime of each mass unit released in the atmosphere and it provides an estimate of the maximum calculated effect of a compound relative to the maximum calculated effect of an equal amount of CFC-11. The ODP concept is widely used to evaluate the potential effects on stratospheric ozone of CFCs and HCFCs (WMO, 1989).

A first approximation of the ODP value can be based on atmospheric lifetime and the number of Cl and Br atoms per molecule (Brown et al., 1989). In general, ODP values approach zero for species with atmospheric lifetimes less than one year.

Tropospheric ozone

The efficiency of organic compounds in the production of tropospheric ozone and the timescale on which the ozone formation takes place, depends on a number of factors:

- the reactivity of the compound and the degradation pathway,
- the meteorological conditions,
- the concentrations of other air pollutants,

Meteorological conditions leading to the highest ozone concentrations during photochemical episodes are characterized by high temperatures, high levels of solar radiation and low wind speeds. Highly reactive compounds, like xylene, olefines or aldehydes, contribute significantly to the ozone peak values. Low reactive species, such as carbon monoxide, methane and higher alkanes, are important for ozone formation in the free troposphere and therefore for the long-term ozone concentrations.

For organic compounds containing *n* C-atoms and *m* H-atoms the upper limit of ozone molecules that can be formed when the molecule is fully oxidized, is, under the most favourable conditions $(n+m)$ ozone molecules. With respect to episodic ozone, Derwent & Jenkin (1990) have suggested the use of a photochemical ozone creation potential (POCP) index as a means of comparing different organic species. The POCP index is a measure of the relative effect on ozone of a unit mass of any organic compound compared to that caused by an equivalent mass of ethylene. By definition, ethylene has always a POCP value of 100. The evaluation of POCP values requires (next to a substantial computer effort) detailed knowledge about the degradation pathway. At present there is no procedure available to estimate the effect on tropospheric ozone when only the basis characteristics of a new substance are known. Further research is needed.

Acidification

During the oxidation of substances containing Cl, F, N or S substituents acidifying components (e.g. HCl, HF, NO_2 and nitric acid, SO_2 and sulfuric acid) may be formed. Following deposition these oxidation products will lead to acidification of the receiving soil or surface water. Analogous to the definition of GWP and ODP an 'Acidification Potential' (AP) can be defined as the number of potential acid equivalents per mass unit compared to the number of acid equivalents per mass unit of a reference compound; sulfur dioxide (SO_2) is proposed as reference gas. The number of potential acid equivalents per molecule is obtained by summation of the number of Cl, F, N and 2xS substituents.

6.2.3 Major accident hazard assessment

Large scale calamities with (new) chemicals can occur during production, transport and storage. A calamity is for example:

- leakage of liquid from a vessel;
- spilling of liquid during bulk transport (accident);
- leakage of gas from a gas-container;
- spilling of chemicals into a stream;
- explosion of (part of) a plant.

Directive 82/501/EEC (EC, 1982) on the major accident hazards of certain industrial activities (Post-Seveso Directive) regulates harmonized prevention of potential major-accidents by means of:

- regulation of industrial activities (listed in Annex I of Directive 82/501) and industrial storage of dangerous substances (Annex II of Directive 82/501) together with their respective threshold quantities;
- notification requirement of existing industrial activities listed in Annexes II and III of Directive 82/501;
- informing the CEC by the Member States of dangerous substances to be added in Annexes II and III of Directive 82/501.

The criteria for indicating dangerous new substances with a potential major-accident are first the dangerous substances criteria according to Annex VI of Directive 79/831/EEC, and secondly the threshold quantities in storage.

The risk that a major accident will occur can be calculated if local conditions are well known. Some methods are described in CPR (1985). Given the lack of data on production method, transportation, and storage, it seems questionable that precise statements can be made regarding major-accident risks. However, it seems feasible to estimate major-accident risks once a certain scenario has been adopted, possibly coupled to the production and use data. The EC has developed a semi-quantitative hazard assessment method directed to potential major-accident hazard, the MEC program (Bello, 1989). Such a program, or a simplified version, would facilitate the identification of new substances with major-accident hazards.

This subject has not yet been incorporated in the regular assessment procedure for new substances at the RIVM, but is still under investigation.

6.2.4 Hazard assessment for waste treatment

The three main routes for the treatment of waste material are:

a. incineration of domestic waste, industrial waste, sludge, etc.,

b. landfilling with the same types of waste,

c. specific treatment as hazardous waste (chemical waste).

ad a.

It is not to be expected that new substances as such will enter the environment, unless they are very stable substances and are considerably volatile. No data are available on evaporation and escape without combustion of substances in incinerators. The risk at incineration would then be the formation of other toxic compounds (e.g. dioxins) out of new substances. The problem involved in this is that the efficiency of conversion into dioxins cannot be estimated. Only the presence of chlorine in the molecule (probably combined with an aromatic structure) could be relevant.

ad b.

Since the new substances occur in products, two aspects are important: the degradation rate and the release rate of the substances. With respect to degradation, a risk evaluation of metabolites could be considered which should be carried out for different situations (e.g. waste water treatment plants, all environmental compartments). Very few data generally are available on the release rate of substances due to, for example, migration, evaporation and/or leaching to permit a reasonable quantitative estimate (which also holds for existing and widely used compounds).

ad c.

Treatment of substances as hazardous waste involves mainly incineration under strict conditions (e.g. high temperature, off-gas treatment) and landfilling at special dumping sites where measures have been taken to prevent contamination of the soil. Thus, proper treatment as hazardous waste means that (new) substances do not enter the environment.

7. FURTHER TESTING REQUIREMENTS

7.1 When and what?

Additional studies may be required at several stages following base set testing:

1. Immediately post-base set:
   - if serious deficiencies are found in the base set a repeat test or an additional test may be deemed necessary
   - in case of a positive result in any in vitro genotoxicity test (section 7.2.5).
   - in case of high predicted hazards or risks.
2. If the quantity of a substance produced or put on the market by a particular notifier reaches a level of 10 tonnes per year or a total of 50 tonnes (level 0^+) it may be deemed necessary to initiate level 1 ($\geq$ 100 tonnes/year) testing. The following level 1 tests may then be required:
   - genotoxicity tests
   - biodegradation tests
   - ecotoxicological tests.

   The conditions at which these tests are required are specified in section 7.2.
3. If the quantity of a substance produced or put on the market by a particular notifier reaches a level of 100 tonnes per year or a total of 500 tonnes, level 1 tests are required.
   The full level 1 test-package is described in Annex VIII of Directive 79/831/EEC (EC, 1979).
   The conditions at which these tests are required are specified in section 7.2.
4. If the quantity of a substance produced or put on the market by a particular notifier reaches a level of 1000 tonnes per year or a total of 5000 tonnes, level 2 tests are required. The conditions at which level 2 tests are required will be discussed in a future report.

It is emphasized here that the need for further studies as recommended below does not imply any recommendation on other aspects of risk management. Risk management is not restricted to demands for further studies.

7.2 Testing strategies

7.2.1 Introduction

Level 1 tests shall be carried out unless it is not technically possible or if it does not appear scientifically necessary (EC, 1979). Scientific reasons to exclude a test from the level 1 set can be based on the results of the hazard identification at level 0, the result of the hazard/risk

assessment at level 0 which includes the exposure factor, and any other additional knowledge of the substance itself or of chemically related substances. In the following testing strategies will be discussed for each level 1 test. It is noted that a special EC Working Group is preparing a strategy document, which will be a guideline for choosing appropriate administration routes in toxicity testing (EC, 1991b).

7.2.2 Fertility test

This test is not required if exposure of workers or of the general public, directly and indirectly via the environment, will be negligible. In all other cases at least a one-generation test need be carried out including a teratogenicity screening. The teratogenicity screening is not required if a full teratogenicity test has been, or will be, carried out. A second generation should be studied if there are equivocal findings in the first one.

7.2.3 Teratogenicity test

This test is not required if exposure of workers or of the general public, directly and indirectly via the environment, will be negligible. In all other cases a teratogenicity test is required unless the teratogenicity screening included in the fertility test was negative. The latter test is considered insufficient for an evaluation of the teratogenic potential of a substance if the substance is structurally related to known teratogens, will probably bioaccumulate (log K_{ow} ≥3, section 4.4.3.2), is classified as toxic or highly toxic, or if a large number of people will be exposed. These predisposing factors are derived from an advice from the Dutch Health Council (HC, 1985b).

7.2.4 Subchronic and/or chronic toxicity test

This test is not required if exposure of workers or of the general public, directly and indirectly via the environment, will be negligible. In all other cases a 90-day test should first be seriously considered. Factors increasing the need for a 90-day test are the following:

a. Exposure pattern

- repeated uptake of the substance by consumers is very likely; examples are substances which are used in food, drinks, household sprays, detergents
- repeated uptake of the substance by workers is very likely; examples are solvents, additives in lubricants and paints
- the margin of safety (MOS) for indirect exposure as predicted by the Assessment System for New Substances (section 5 and 6) is low

b. Toxicological profile

- serious lesions or irreversible lesions in the 28-day test; it should be realized also that a 28-day test can be too short to reveal the irreversibility of a lesion
- certain indications in the 28-day test that the substance may exert a certain serious effect which needs a longer exposure period for expression or which has not yet been investigated properly; examples are suspected immunotoxicity in case of changes in circulating leucocytes and depletion of cells in lymphoid tissues, suspected neurotoxicity on the basis of persisting behavioral changes, suspected renal dysfunction on the basis of urinalysis; such situations will also indicate which special studies need be performed
- a very low, or absent, no-observed-adverse-effect level
- a plain relationship in chemical structure between the substance and others which have been proved dangerous; such a relation may also reveal which special studies need be performed such as extra attention to an organ or tissue expected to be a target or additional biochemical tests known to be particularly suitable to reveal an expected lesion; an example is a special investigation into thyroid function in case of bromides.

The need for a chronic test usually follows from the result of the subchronic test. In case carcinogenicity testing is considered, simultaneous chronic toxicity testing can replace a subchronic test.

7.2.5 Genotoxicity tests

7.2.5.1 *Introduction*

Within the context of the 6th Amendment (EC, 1979) (and the proposed 7th Amendment (EC, 1990c) is required that the genotoxic potential of new substances is investigated to an extent which permits hazard identification. A stepwise, tonnage triggered approach to testing for genotoxicity is indicated in the Annexes to the Directive (see Annex IIIa). However, there are several test methods, of differing sensitivity and often covering both in vitro and in vivo systems that can be used at various steps. Therefore, a strategic approach, concerning primarily hazard identification and not quantitave risk assessment of new chemicals, has been developed by a working group of the EC. This guideline is meant to be used by Member States when evaluating notification dossiers and giving advise to notifiers on testing requirements. The objective of this strategy is to obtain the required amount of information for adequate hazard assessment at each level of supply from a minimal number of well conducted studies selected on a scientific basis. This EC strategy on genotoxicity testing of new chemicals (draft document XI/449/91-rev3) has served as a basis for the following paragraphs.

7.2.5.2 *Definitions*

For definitions: see section 4.3.5.1.

7.2.5.3 *Adequacy and interpretation of the results*

It is advocated that a minimum number of studies should be used to investigate the possible genotoxicity of chemicals. Therefore it is essential that the tests are conducted according to rigorous protocols in order to maximize the potential for detecting a genotoxic response. It is preferred that tests are performed in accordance with the methods of Annex V of the Directive 79/831 and/or in compliance with the OECD guidelines.

In all tests (in vitro and in vivo) for genotoxic effects, test substance concentrations/doses and way/route of exposure should be selected to ensure sufficient exposure of the target cells within the constraints imposed by toxicity, osmolality, pH etc.
For example, in widely used tests for genotoxic effects in somatic cells in vivo, the target tissue is bone marrow. Bone marrow is readily accessible to blood borne substances and the route of exposure should be chosen to ensure that significant absorption of the administered chemical, or its metabolites, into the blood stream occurs. Thus, if it is known or suspected that the test substance is poorly absorbed following oral administration, other potential routes of administration are to be considered.
Indicative evidence that exposure of the target tissue has occurred is of particular importance in the evaluation of tests if the results are negative.
Very reactive substances, which are positive in vitro, may react with proteins and water in in vivo test systems and thus be rendered inactive to many tissues. They are most likely to express their genotoxic potential at the initial site of contact and represent a particular threat to the respiratory tract, upper gastrointestinal tract and skin.

Substances giving equivocal results should normally be retested immediately, perhaps varying the conditions. Wherever possible, clear results should be obtained for one step in the strategic procedure before going on to the next.
The results of the genotoxicity assays need to be evaluated in the light of other available information and may include chemical structure, physico-chemical properties and toxicokinetics. Chemicals with a structure of concern (i.e. they share structural characteristics with known mutagens/carcinogens) may be required to undergo further testing earlier than those without such structural characteristics.

7.2.5.4 Practical guidance

The basic principle underlying the strategic testing of a new chemical substance for potential genotoxicity is that the database is gradually extended, in a scientific manner, as the amount of substance supplied in EC increases (Annex IIIa), so that the objectives outlined above are achieved.

When genotoxic effects are observed or indicated further testing may be required in advance of tonnage triggers (Annex IIIb). Tests need not to be performed if it is technically not possible or if they are not considered necessary in the light of current scientific knowledge.

Limited announcements/mini-notification

For substances supplied at a volume of >100 kg per year and <1 tonne per year, a gene mutation test in bacteria should normally be conducted (usually an Ames test, section 4.3.5.3). An alternative in vitro test may be considered appropriate in some cases.

Positive results will need, normally, immediate follow up in a second test according to the strategy outlined below. However, this follow up, and further testing, will be influenced by the potential for human exposure (the use pattern and the predicted future supply levels).

Base set (1 tonne per year or more)

Two tests, normally to be conducted in vitro, are required: a gene mutation test in bacteria and a non-bacteriological test detecting chromosomal aberrations or both chromosome aberrations and gene mutations.

The bacteriological test should be a Salmonella/microsome reverse mutation assay (section 4.3.5.3) and the result of this test should be obtained before instigating the non-bacteriological test.

If the bacteriological test is negative the second assay should be a cytogenetic assay for chromosome aberrations using metaphase analysis (section 4.3.5.3).

If the bacteriological test is positive the second test should be preferably a mammalian cell gene mutation assay, using mouse lymphoma cells (L5178Y), in which the assessment of both large and small colonies is recommended. Alternatively both the HPRT gene mutation assay and the in vitro chromosomal aberrations assay in mammalian cells may be conducted (see for details on test systems section 4.3.5.3).

Substances which by virtue of e.g. their physico-chemical properties, chemical reactivity or toxicity cannot be tested by the above mentioned in vitro tests are considered on a case by case basis. Chemicals with significant bactericidal activity may need to be investigated in a mammalian gene mutation assay in vitro first. Insoluble substances, with structures of concern, could be subjected to appropriate extraction procedures, and the extracts tested (as is done

for medical devices: ISO, 1991).

There may be rare occasions that substances are incompatible with in vitro testing and base set testing in vivo is indicated: e.g. if it is not possible to perform a satisfactory test in vitro and there are structural indications of genotoxic potential of the substance or its metabolites.

Further testing: general principles (Annex IIIb)

Further testing for genotoxicity is triggered either by positive previous test results or by attainment of tonnage thresholds defined in the Directive. The chemistry of the substance, data on analogous substances, data on biotransformation and potential for human exposure will also influence the timing and pattern of further testing.

Further testing: substances which are negative in both base set tests

The timing and extent of further testing will depend largely on the human exposure likely to occur and will not usually be initiated before the supply level reaches 100 tonnes per year (500 tonnes cumulative).

However, further testing may be required earlier as soon as supply levels reach 10 tonnes per year (50 tonnes cumulative), e.g. if exposure of human beings is likely. In addition, a modified reverse mutation test in Salmonella (Prival and Mitchell, 1982) for azo dyes with structures of concern will be required immediately post base set.

If it can be demonstrated to the satisfaction of the competent authority that human exposure is negligible then further testing may be reduced, deferred, or omitted.

A mammalian cell gene mutation assay is thought particularly appropriate to follow up two negative base set in vitro tests. If the third test is also negative, the performance of a fourth test may be considered at 100 or 1000 tonnes per year (500 or 5000 tonnes cumulative). This test should preferably be conducted in vitro using a metabolizing system different from the conventionally used S9 (e.g. freshly isolated hepatocytes). Consideration may be given at this stage to the investigation of aneuploidy.

If any post base set in vitro assay is positive, immediate testing in vivo is indicated.

Further testing: substances for which the base set in vitro cytogenetics test was positive

Immediate post base set testing in vivo is indicated. It should be ensured that the protocol used takes into account the criteria outlined under the paragraph "adequacy and interpretation of the results" (section 7.2.6.3). It is recommended that the first test conducted in vivo should normally be an assay for chromosomal damage in bone marrow (a micronucleus or a metaphase analysis in mice or rats may be used).

If the first in vivo study is negative, a second test using a different target tissue (somatic) should be performed immediately at 10 tonnes per year (50 tonnes cumulative) or 100 tonnes

per year (500 tonnes cumulative), depending on the potential of human exposure. The in vivo/in vitro liver UDS assay or mouse spot test may be used. Alternatively, an in vivo study to detect covalent DNA binding (in several tissues) in an appropriate species could be used.

Further testing: substances for which the bacterial gene mutation test was positive

These substances usually will also have been investigated using the mouse lymphoma TK+/- test. If the mouse lymphoma test or either one of the alternative tests is positive, the approach described in the aforementioned paragraph should be followed i.e. immediate post base set testing in vivo should be instigated. It is recommend that the first test conducted in vivo is a mouse bone marrow study.

If the mouse lymphoma test or both of the alternative tests are negative, investigation in vivo is, nevertheless, necessary for thorough investigation of the potential for the effect indicated by the positive bacteriological test to be expressed in vivo. However, in this case immediate testing will only be required for substances to which significant human exposure (particularly of consumers) occurs. For other substances, testing in vivo can normally be deferred until 10 tonnes per year (50 tonnes cumulative) (see Annex IIIb).

Further testing: substances which are positive in an in vivo test for genotoxic effects in somatic cells

Substances which are positive in tests for genotoxic effects in somatic cells in vivo should be considered with regard to their potential to affect germ cells, normally not later than at 100 tonnes per year (500 tonnes cumulative). However, further investigation will be compulsory when the potential extent of human exposure gives cause for concern.

Assays specially designed to investigate transmitted effects to the progeny like the specific locus test and the heritable translocation test use very high or extreme numbers of animals. Therefore, they are rarely used and will not normally be asked for in the case of industrial chemicals. Usually the dominant lethal assay is used to detect genetic damage, which may have occurred at any stage of the germ cell maturation. However, only effects that are lethal to the zygote are detected. Other tests are available which investigate effects in germ cells in vivo, yet give no indication on the likelihood of transmission.

In practice, it can be assumed that when a chemical is demonstrated to be a somatic cell mutagen that can reach the germ line (eg. by toxicokinetic studies, toxicity tests, germ line adduct studies etc.) it is capable of causing heritable damage.

7.2.6 Aquatic toxicity tests

Further testing on aquatic organisms (algal test, prolonged toxicity tests on fish and Daphnia)

depends on the results of the hazard and risk evaluation at level 0 (sections 5 and 6). If more information on, for example, use, function, life cycle and/or emission rates of the substance has become available, this risk assessment must be updated. Discriminating parameters are: the PEC/NEC-ratio and the probability of adverse effects (section 6.2.1.3), persistence (chemical degradability and biodegradation; sections 4.4.1 and 4.4.2), and bioaccumulation potential (section 4.4.3). Table 6 shows the relation between these parameters as determined in the hazard and risk evaluation and the need for further research as recommended in general by RIVM. It should be stressed that any rigid decision scheme would not be appropriate and that the criteria in table 6 can never be absolute: depending on the available data test requirements may change.

As can be observed from table 6 the higher the estimated risk at level 0, the sooner and the more tests will be asked. In addition, persistency and bioaccumulating potential are factors which increase the need for long-term tests. The class "unknown" contains poorly soluble substances which do not show any adverse effects in acute toxicity tests. In case a choice has to be made between the prolonged toxicity test on fish and the prolonged reproduction toxicity test on waterfleas, preference is usually given to the species which has shown to be the most sensitive. On the basis of structure-activity relationships or in case of
indications of genotoxicity, preference can, however, be given to the waterflea test. If there is no apparent or an unknown difference in sensitivity, preference is also given to the test with waterfleas which covers the complete life cycle.

7.2.7 Terrestrial toxicity tests

Toxicity tests on terrestrial organisms are not submitted at tonnage level 0. At level 1 it is possible to ask for a test on higher plant and earthworm. These tests should be submitted unless the substance clearly cannot reach the soil compartment.

7.2.8 Biodegradation tests

Like the base set biodegradability tests, further tests also pertain to the aquatic aerobic environment. The base set tests allow a distinction between "readily biodegradable" and "not readily biodegradable" substances. Further testing, which may already be required at tonnage level 0, should categorize the substances in the latter group as "inherently (potentially) biodegradable" or "not degradable". A simulation test is designed to permit the final evaluation of the biodegradability of a substance at "field conditions". Further testing may also include anaerobic, photo-, or soil degradation tests.

Table 6: General criteria for further aquatic toxicity testing[1]

P[2]	Readily degradable	Bioaccumulation potential	Testing requirements[3]	
			0+	1
P < 0.20	y	y/n	-	a
	n	y/n	-	a,c
0.20 ≤ P < 0.50	y	y/n	a,d	a,d
	n	n	a,b,d	a,c,d
	n	y	a,c,d	a,c,d
P ≥ 0.50	y	y/n	a,c,d	a,c,d
	n	n	a,c,d[4]	a,c,d[4]
	n	y	a,c,d[4]	a,c,d[4]
Unknown	y	n	-	-
	n	n	a,b[4]	a,b[4]
	n	y	a,b[4]	a,b[4]

[1] - = no further testing recommended
y = yes
n = no

[2] Probability that PEC ≥ NEC-eco

[3] a = alga, growth inhibition test
b = one prolonged toxicity test
c = two prolonged toxicity tests
d = activated sludge, respiration inhibition test

[4] in certain cases immediate post-base set testing may be recommended, e.g. if P ≥ 0.80 or in case of widespread exposure.

A special EC Expert Group has outlined a strategy for biodegradability testing. An overview of this strategy is presented in Annex IIIc (EC, 1986c). It is, however, recommended to refer to the original document.

7.2.9 Bioaccumulation tests

As described in section 4.4.3.2, a log K_{ow} value of 3 is regarded as a cut off value for further testing of bioaccumulation in fish for substances which are not readily biodegradable, do not ionize in water, are not (organo-)metallic, and have a molecular mass below 700 dalton. Further testing requirements for substances which do not meet these criteria should be established on a case by case basis, including for example structure-activity considerations.

8. INTERNATIONAL DEVELOPMENTS

8.1 7th Amendment

Directive 79/831/EEC was in fact the 6th amendment to Directive 67/548/EEC. It was enforced in Member States since September 1981. More than 600 new substances have been notified in the EC since then. In addition, approximately 450 limited announcements have been submitted in The Netherlands alone. On the basis of the experience gained a 7th amendment is in preparation in order "both to rectify any anomalies which have come to light since 1981 and also to improve the efficiency of implementation" (EC, 1990c). Several changes which are significant with respect to the assessment of new substances will be highlighted below.

The EC Directive 79/831/EEC already requires an evaluation of the potential hazards/risks of notified substances to man and the environment. This objective is now explicitly mentioned in the first article of the new proposal. As a consequence the EC Member States have outlined common principles and a stepwise procedure for an environmental hazard assessment, which will be discussed in section 8.2. In addition, competent authorities may ask for further information, supplementary testing and verification/confirmatory tests concerning the substance or its degradation products (metabolites), if it can be shown to be necessary for the evaluation of the hazard/risk. These requests and the point in time at which these requests are made, are then more directed by the assessment of the substance than by the amount notified.

The definition of "substance" has been clarified in order to ensure that the notifiers notify substances as pure as possible without solvents and removable contaminants.

New definitions have been proposed for a "classification of sensitizing" and for "carcinogenic, mutagenic and toxic for reproduction". The definition of "dangerous for the environment" has been slightly modified and a symbol for this classification introduced.

The system of limited announcements in each Member State for substances put on the market in quantities less than one tonne will be replaced by one single harmonized reduced notification procedure with a very limited dossier for substances in quantities between 10 and 100 kg/year and a limited dossier for substances in quantities between 100 and 1000 kg/year. Such a reduced notification, once submitted, will allow free circulation throughout the EC.

In the new proposals the review period for notifications will be extended from 45 to 60 days

and there will be no further deadline for the assessment of the acceptability of the notification. The review period for harmonized limited announcements will be 30 days.

New elements in the base set are proposed to be a prescreening test for teratogenicity, a growth inhibition test for algae, and an adsorption/desorption test. Reduced base sets have been outlined for harmonized limited announcements.

8.2 A harmonized EC assessment

The EC has produced a document describing common principles and a stepwise procedure for the environmental hazard assessment of new substances (EC, 1990d). The model described has been accepted by the Member States and was further discussed at an EC Workshop in October 1990 (EC, 1990e). This work will continue, shifting the main emphasis from the work on environmental hazard assessment to the development of harmonized approaches with regard to the assessment of hazards to human beings exposed directly (consumers, workers, human beings exposed via ambient air) and indirectly (human beings exposed through the food chain). Toxicity testing strategies will be developed for mutagenicity, reproductive toxicity, and inhalation toxicity (EC, 1991c).

The harmonized EC model estimates a "worst-case initial environmental concentration" (IEC):

$$IEC = E \times 10\ (100 - P)/V_o$$

with IEC = initial environmental concentration [mg/l]
E = emission rate [kg/day]
V_o = volume of water containing E [m^3/day]; default is 2000 m^3/day
P = % removed from the water compartment by degradation, adsorption or volatilization; these removal rates should be estimated on the basis of physico-chemical properties and the results of ready biodegradation tests.

Substances classified as "dangerous for the environment" and substances for which a potential environmental hazard is suspected on the basis of use pattern, other classifications, persistency, or chemical structure, will be selected for a hazard assessment in which acute aquatic toxicity data are compared to the IEC:

$$\text{Hazard ratio} = [\text{lowest acute toxicity } L(E)C_{50}]/IEC$$

For the initial hazard assessment a hazard ratio of below 1000 triggers further assessment, including the need for further testing, the need for more information on exposure and the need for regulatory measures. In the Dutch Assessment System for New Substances the trigger can be either the ratio of the predicted environmental concentration (PEC) and the extrapolated no-effect concentration for the aquatic ecosystem (NEC-eco) or the probability that the PEC exceeds the NEC-eco (chapter 5).

9. DISCUSSION

This report presents to the reader a fairly detailed overview of the initial assessment of the hazards and risks of new chemicals at base set level to man and the environment. It is a reflection of the experience gained at the RIVM in this area. The authors of this report do not claim to have covered all aspects of the assessment of new chemicals nor to have answered all the questions the reader may have. Rather, they hope that this report will present clarity on the way new chemicals are assessed at the RIVM and that it will be a starting point for further research and discussions. Updates of this report are envisaged as well as publications on the post base set assessment.

The initial hazard and risk assessment of new substances involves the integration of exposure and effects data and, as far as is possible, the estimation of uncertainty in these data (see also sections 5.1.5 and 6.2.1.4). This uncertainty arises due to variability in the environment and in human conduct on the one hand, and due to limitations in the model equations and data used on the other hand. On the basis of the overall assessment the risk manager may decide to act at once or to ask for a refinement of the assessment. In the latter case, more data are usually needed to reduce the uncertainty in the predictions. This reduction can be effectuated by expanding the data on emission, environmental fate and/or toxicity. Before discussing these options it should, however, be borne in mind that other factors than those presently covered by an exposure-toxicity comparison may trigger actions from the regulator. Examples are: persistency per se, ozone depletion potential, waste problems such as dioxin formation at incineration or the absence of waste treatment possibilities, special hazards to consumers, and analogy with acknowledged hazardous substances. In fact, the three new substances proposed for a ban by The Netherlands since the enforcement of the Chemical Substances Act, i.e. DBB (dibutyl tin borate), Ugilec 121 (mixture of chlorobenzyl-chlorotoluene isomers), and DBBT (mixture of bromobenzyl-bromotoluene isomers) have primarily been regulated on the basis of their analogy with organotins (DBB) and PCB's (Ugilec 121, DBBT). Another aspect which constantly should be in the mind of both the risk assessor and the risk manager is the cost-benefit analysis, in terms of risk to man and the environment, of the replacement of an existing chemical by a new chemical.

A reduction of the uncertainty in the initial exposure assessment may be effectuated by additional information on the life cycle of the substance: use and function of the substance, tonnage level per plant or number of processing plants, processing conditions (e.g. water usage, losses, processing capacity per unit of time), specific data on the site of production and

processing (e.g. the characteristics of the waste water treatment plant, effluent dilution factors), concentrations in products, and migration rates. Obviously, the notifier usually is the most important source of this kind of data, but data on comparable substances may also help. A reduction in the uncertainty in environmental fate may be achieved by more accurate data on partition coefficients (soil-water, air-water) and (bio)degradation in air, water, and soil, as these are the dominating parameters in the environmental distribution.

A reduction of the uncertainty in the effect data may be achieved by further testing. A carefully chosen testing strategy is an asset here. This report has discussed this subject in detail (chapters 7). Constant awareness for improvements in test guidelines and communicating the experience gained to the experts involved in the development and updating of test guidelines is equally important. The test guidelines should be flexible enough - as well as the notifiers and the Competent Authorities - to encourage test laboratories to resolve emerging problems and questions as much as possible. Merely following the minimum requirements of guidelines is certainly the opposite of what scientific research should be. Moreover, it often leads to further testing requirements from the Competent Authorities which could have been avoided if a test had been expanded slightly more. Another observation related to further testing requirements is that the present close connection between tonnage levels and further testing requirements, as conceived in the EC Directive, gradually may be replaced by an approach linking further testing requirements more directly to the estimated degree of hazard or risk as confidence in the methods of quantitative hazard/risk assessment will be growing.

It is noted here that the Assessment System for New Substances is capable to at least partly quantify the uncertainty in the exposure assessment but still falls short in doing this for the effect data. Taking for a fact the uncertainty factors usually employed in the derivation of acceptable levels for man from experimental animals data, it can be assumed that the overall uncertainty in the effect data is at least as high as the uncertainty in the exposure assessment. This area of scientific research is still full of opportunities.

One important question currently under debate at the RIVM is: which human being should be protected? At the moment a hazard quotient is calculated for an adult living in a standard environment near the source of emission of a particular substance and inhaling air, drinking tap water and consuming food all completely contaminated by this substance. Alternatively, hazard quotients could be calculated for various subgroups at risk. Further model analysis will be carried out to answer this question.

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ANNEX I
EXPLANATION OF ABBREVIATIONS AND DEFINITIONS

acute toxicity test	toxicity test serving to study the effects occurring in a short time following the administration of a single dose or multiple doses given within this short time period
alopecia	absence of hair from areas where it normally grows
Annex II	annex to Directive 79/831/EEC containing symbols and indications of danger for the label
Annex V	annex to Directive 79/831/EEC containing the test guidelines for the base set
Annex VI	annex to Directive 79/831/EEC containing criteria for classification and labelling
Annex VIII	annex to Directive 79/831/EEC containing the additional information and tests required post base set
AP	Acidification Potential
atrophy	the wasting away of an organ or tissue due to degeneration of cells
auxotroph	mutated strain requiring specific growth factors not required by the parent organism
base set	the minimum data set required by law for notifications of new substances with a marketing level of 1 tonne/year or more
basophil	white blood cell (leucocyte) easily stained by basic aniline dyes
BCF	Bioconcentration Factor: the ratio of the test substance con-

	centration in (part of) an organism (e.g. fish, plant) to the concentration in a medium (e.g. water, soil) at steady state
BOD	Biological Oxygen Demand
buffer capacity	resistance against changes in pH when acid or alkali is added
CAS	Chemical Abstracts Service
CFC	ChloroFluoroCarbon
chemosis	swelling of the conjunctivae
chronic toxicity test	a study in which animals are observed during the whole lifespan and in which exposure to a substance takes place over the whole observation time or a substantial part thereof
COD	Chemical Oxygen Demand
cornea opacity	transparant epithelium and connective tissue of the eye overlying iris and lens
critical effect	is the first effect, considered to be adverse, appearing when the dose of a substance is increased
CSA	Chemical Substances Act
CSB	Chemical Substances Bureau ("the Bureau"): the competent authority with respect to the implementation of the Chemical Substances Act on new substances
desquamation	the process in which the outer layer of the epidermis of the skin is removed by scaling
DI	Daily Intake
DOC	Dissolved Organic Carbon

dose	the amount of a chemical administered
dose-response assessment	establishing the relationship between the dose and the incidence of adverse effects in a particular group of test organisms and, through extrapolation, in a whole population
DWE	Dose Without Effect: highest exposure level without adverse effects
EC_{50}	median Effective Concentration: 1. the concentration resulting in a 50% change in a parameter (e.g. algal growth) relative to the control 2. the concentration at which a particular effect (e.g. Daphnia immobilization) is observed in 50% of the organism population relative to the control
ECETOC	European Chemical Industry Ecology and Toxicology Centre
E(E)C	European (Economic) Community
effect	the extent of biological changes
EINECS	European Inventory of Existing Chemical Substances: lists all chemical substances defined as "existing" prior to 18 September 1981
erythema	increased redness of skin or eye by dilatation of the blood capillaries
FCA	Freund's Complete Adjuvans
fibrosis	increased amount of connective tissue in an organ or tissue
freon	chlorofluorocarbon (CFC, halon)
GLP	Good Laboratory Practice: a set of rules describing how a laboratory should work, how it should be organized and how it can produce valid data; GLP principles are described by OECD

GMP(T)	Guinea Pig Maximization (Test)
GWP	Global Warming Potential
hardness (of water)	property of water indicating the total amount of calcium, magnesium and barium
hazard	the potential of a substance to cause adverse effects at a particular degree of exposure [Dutch: gevaar, risico (in algemene zin)]
hazard assessment	determination of the potential of a substance to cause adverse effects qualitatively or quantitatively by comparing estimated exposure levels to toxicity values
hazard identification	the process of determining whether exposure to a substance can cause an increase in the incidence of an adverse effect
HCFC	HydrogenChloroFluoroCarbon
hydropic change	severe cellular swelling producing small clear vacuoles or single large vacuoles by intracellular accumulation of water
hyperplasia	the increased production and growth of normal cells in a tissue or organ
hypertrophy	increase in the size of a tissue or organ brought about by the enlargement of cells rather than by cell multiplication
IC_{50}	median Inhibitory Concentration: the concentration resulting in a 50% inhibition of growth relative to the control
incidence rate	the fraction of a population (e.g. a group of experimental animals) showing a particular effect in a given period of time
IUPAC	International Union of Pure and Applied Chemistry
karyotype	description of the number and structure of a chromosome set

	of an individual or species
K_{ow}	octanol-water partition coefficient
LC_{50}	median Lethal Concentration: a statistically derived concentration that can be expected to cause death in 50% of animals exposed for a specified time
LD_{50}	median Lethal Dose: statistically derived single dose that can be expected to cause death in 50% of dosed animals
LED	Lowest Effect Dose: the lowest exposure level in one test at which the most critical effect is occurring
limited announcement	notifications of certain substances for research and development purposes and of substances with a production, importation or marketing volume below 1 tonne/year
LOAEL	Lowest-Observed-Adverse Effect Level: the LED for the most critical test
macrophage	white blood cell removing foreign particles from connective tissue, blood, and lymph by phagocytosis (eating up)
metaphase	stage in the cell division process
metaplasia	proliferation in which one type of differentiated cell is substituted for another type of differentiated cell
MOS	Margin Of Safety: the ratio of the estimated daily intake of man to the DWE
NEC-eco	No-Effect Concentration for a particular ecosystem
necrosis	death of some or all of the cells in an organ or tissue
neuropathy	any disease of the peripheral nerves

neutrophil	white blood cell (leucocyte) easily stained by neutral dyes
NOAEL	No-Observed-Adverse-Effect Level: the DWE for the most critical test
NOEC	No-Observed-Effect Concentration: highest concentration without adverse effects
NOEL	No-Observed-Effect Level: the exposure level without any effect
ODP	Ozone Depletion Popential
OECD	Organization for Economic Cooperation and Development
oedema	excessive accumulation of fluid in the body tissue
PEC	Predicted Environmental Concentration
photodegradation	the reaction of a compound with (hydroxyl-, ozone-, nitrate-) radicals produced by the action of light
photolysis	the breakdown of a compound as a result of irradiation by light
PMN	Pre-Marketing Notification
POCP	Photochemical Ozone Creation Potential
QA	Quality Assurance: internal laboratory control system to ascertain that tests are in compliance with GLP principles
QSAR	Quantitative Structure-Activity Relationship
response	the incidence rate of effects
risk	the probability of a substance to cause adverse effects [Dutch: risico (in statistische zin)]

risk assessment	determination of the relation between the predicted exposure and adverse effects using probability functions
risk management	decisions aiming at measures which are appropriate at least to diminish significantly the risk of a substance in the environmental compartments of concern
RIVM	National Institute of Public Health and Environmental Protection (Dutch: RijksInstituut voor Volksgezondheid en Milieuhygiëne)
SAR	Structure-Activity Relationship
SCE	Sister Chromatid Exchange
semi-static aquatic toxicity test	a test in which the test solutions are periodically renewed
sensory	relating to the senses, i.e. the input division of the nervous system
sixth amendment	Directive 79/831/EEC, the EC notification Directive on new substances
SOP	Standard Operating Procedure; description of all important laboratory operations
spongiosis	spungelike swelling of skin
steady state	a condition of total equilibrium
structural alert	element in a chemical structure known to be associated with genotoxicity or carcinogenicity
subacute toxicity test	toxicity test serving to study the effects produced by a substance when administered in repeated doses or continuously over a period of up to about 28 days
subchronic toxicity test	toxicity test serving to study the effects produced by a sub-

	stance when administered in repeated doses or continuously over a period of up to about 90 days
TAC	Toxicology Advisory Centre
taxon (plural: taxa)	general term for a taxonomic group, i.e. a group of organisms classified according to their resemblances and differences
teratogenicity	the occurrence of irreversible structural or functional changes or defects in an embryo or foetus
threshold dose	the lowest exposure level in one test at which the most critical effect is occurring
TOXBANK	data bank of the RIVM containing data and evaluations of chemical substances
UDS	Unscheduled DNA Synthesis
US	United States
vasoactive mediators	agents affecting the diameter of blood vessels
WWTP	Waste Water Treatment Plant

ANNEX II

EC COMMENTS REGARDING THE USE OF R48

(EC, 1990c)

"Use of this risk phrase refers to the specific range of biological effects within the terms described below (..) For application of this risk phrase serious damage to health is to be considered to include death, clear functional disturbance or morphological changes which are toxicologically significant. It is particularly important when these changes are irreversible. It is also important to consider not only specific severe changes in a single organ or biological system but also generalized changes of a less severe nature involving several organs, or severe changes in general health status.

When assessing whether there is evidence for these types of effects reference should be made to the following guidelines:

1. Evidence indicating that R48 should be applied
   a. Substance related deaths
   b. i) major functional changes in the central or peripheral nervous systems, including sight, hearing and the sense of smell, assessed by clinical observations or other appropriate methods (e.g. electrophysiology).
      ii) major functional changes in other organ systems (for example the lung).
   c. any consistent changes in clinical biochemistry, haematology or urinalysis parameters which indicate severe organ dysfunction. Haematological disturbances are considered to be particularly important if the evidence suggests that they are due to decreased bone marrow production of blood cells.
   d. severe organ damage noted on microscopic examination following autopsy.
      i) widespread or severe necrosis, fibrosis or granuloma formation in vital organs with regenerative capacity (e.g. liver).
      ii) severe morphological changes that are potentially reversible but are clear evidence of marked dysfunction (e.g. severe fatty change in the liver, severe acute tubular nephrosis in the kidney, ulcerative gastritis).
      iii) evidence of appreciable cell death in vital organs incapable of regeneration (e.g. fibrosis of the myocardium or dying back of a nerve) or in stem cell populations (e.g. aplasia or hypoplasia of the bone marrow).

The above evidence will most usually be obtained from animal experiments. When considering data derived from practical experience special attention should be given to exposure levels.

2. Evidence indicating that R48 should not be applied.

The use of this risk phrase is restricted to "serious damage to health by prolonged exposure". A number of substance-related effects may be observed in both humans and animals that would not justify the use of R48. These effects are relevant when attempting to determine a no-effect level for a chemical substance. Examples of well documented changes which would not normally justify classification with R48, irrespective of their statistical significance, include:

a. clinical observations or changes in body weight gain, food consumption or water intake, which may have some toxicological importance but which do not, by themselves, indicate "serious damage";
b. small changes in clinical biochemistry, haematology or urinalysis parameters which are of doubtful or minimal toxicological importance;
c. changes in organ weights with no evidence of organ dysfunction;
d. adaptive responses (e.g. macrophage migration in the lung, liver hypertrophy and enzyme induction, hyperplastic responses to irritants). Local effects on the skin produced by repeated dermal application of a substance which are more appropriately classified with R38 "irritating to skin";
e. where a species-specific mechanism of toxicity (e.g. by specific metabolic pathways) has been demonstrated.

ANNEX IIIa

STRATEGY ON GENOTOXICITY TESTING I

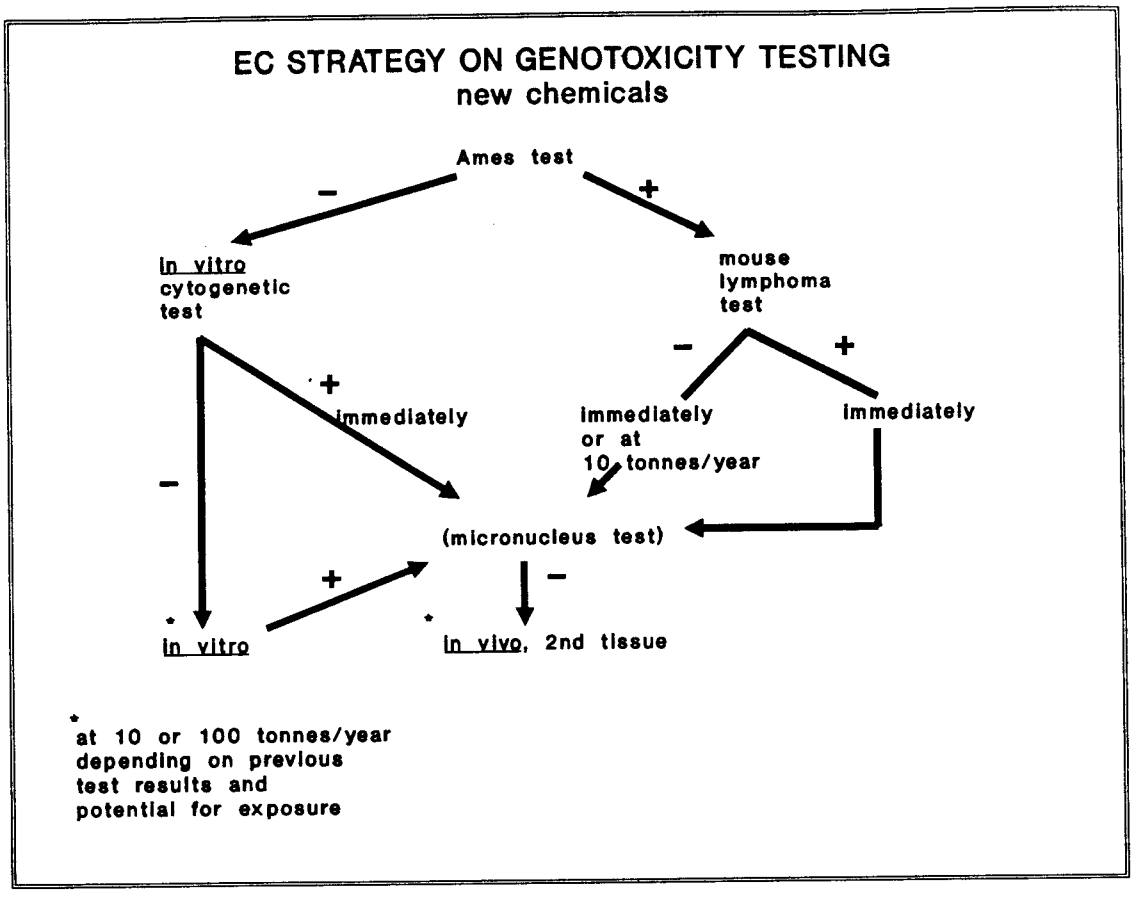
EC, 1979

EEC COUNCIL DIRECTVE 79/831/EEC
genotoxicity testing

LEVEL 0 1-10 tonnes/year	TECHNICAL DOSSIER (ANNEX VII) (Base Set) Two tests: • Gene mutation test in S.typhimurium or E.coli • Chromosomal aberration test in mammalian cells (in vitro)
LEVEL 1 10-100 tonnes/year optional >100 tonnes/year obligatory	ANNEX VIII Four tests: • Two tests of the base set plus • Gene mutation test in eukariotic cells • Chromosomal aberration test in vivo or in vitro
LEVEL 2 > 1,000 tonnes/year	ANNEX VIII Long-term carcinogenicity

ANNEX IIIb

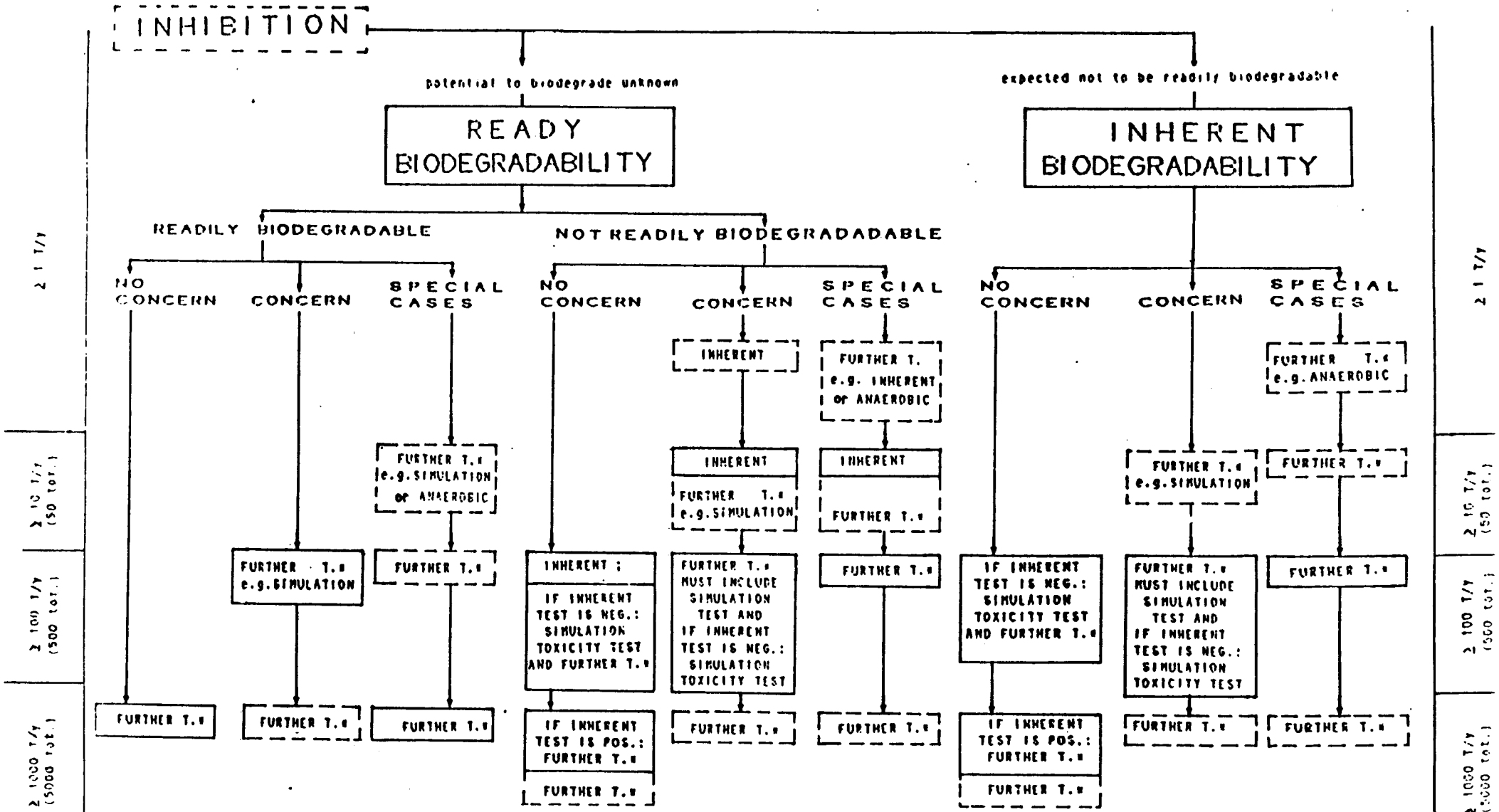
STRATEGY ON GENOTOXICITY TESTING II



ANNEX IIIc

STRATEGY ON BIODEGRADATION TESTING

(EC, 1986c)



[ ] = optional ☐ = to be carried out

* further testing also includes e.g. anaerobic, photo- or soil degradation tests.